A Novel Rabies Vaccine Based on a Recombinant Parainfluenza Virus 5 Expressing Rabies Virus Glycoprotein

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Untreated rabies virus (RABV) infection leads to death. Vaccine and postexposure treatment have been effective in preventing RABV infection. However, due to cost, rabies vaccination and treatment have not been widely used in developing countries. There are 55,000 human death caused by rabies annually. An efficacious and cost-effective rabies vaccine is needed. Parainfluenza virus 5 (PIV5) is thought to contribute to kennel cough, and kennel cough vaccines containing live PIV5 have been used in dogs for many years. In this work, a PIV5-vectorized rabies vaccine was tested in mice. A recombinant PIV5 encoding RABV glycoprotein (G) (rPIV5-RV-G) was administered to mice via intranasal (i.n.), intramuscular (i.m.), and oral inoculation. The vaccinated mice were challenged with a 50% lethal challenge dose (LD50) of RABV challenge virus standard 24 (CVS-24) intracerebrally. A single dose of 10^8 PFU of rPIV5-RV-G was sufficient for 100% protection when administered via the i.n. route. The mice vaccinated with a single dose of 10^8 PFU of rPIV5-RV-G via the i.m. route showed very robust protection (90% to 100%). Intriguingly, the mice vaccinated orally with a single dose of 10^8 PFU of rPIV5-RV-G showed a 50% survival rate, which is comparable to the 60% survival rate among mice inoculated with an attenuated rabies vaccine strain, recombinant LBNSE. This is first report of an orally effective rabies vaccine candidate in animals based on PIV5 as a vector. These results indicate that rPIV5-RV-G is an excellent candidate for a new generation of recombinant rabies vaccine for humans and animals and PIV5 is a potential vector for oral vaccines.

As one of the zoonotic diseases, rabies virus (RABV) infection leads to rabies in warm-blooded animals, including humans, and is characterized by acute encephalitis at the early phase and fatality at the later stage without postexposure treatment (1). Approximately 55,000 human deaths caused by rabies are reported annually, with most of these cases occurring in developing countries (2, 3). Stray dogs, wild carnivores, and bats are the natural reservoirs of field RABV, and these rabid carriers are a public health risk to humans and domestic animals. Human rabies occurrence is largely attributed to the bite of stray dogs in developing countries, where vaccination of animals is limited, especially in rural areas (3, 4).

Vaccination is the most effective method of preexposure treatment against RABV infection and has been used in both humans and reservoir animals. For the postexposure treatment, multiple inoculations of inactivated cell culture vaccines and injection of immunoglobulin are utilized together to prevent the development of rabies. However, the rabies vaccine immunization and immunoglobulin treatment are relatively expensive for families in rural or remote areas of developing countries (5). Vaccinating stray dogs is a potential cost-effective strategy to prevent RABV infection as well. Therefore, an efficacious and cost-effective vaccine is needed. To vaccinate stray dogs, needle-free vaccination, such as oral immunization, would be ideal.

Currently, killed rabies vaccines are prepared from chicken embryo, BHK, or Vero cells and are available for human use and for pet animals via intramuscular (i.m.) injection (6). Preexposure rabies vaccines are routinely administered by three successive injections of inactivated vaccines. For rabies prevention in domestic and wild animals, live attenuated rabies vaccines (strain SAD- and ERA-based modified live rabies vaccines) and recombinant rabies vaccines based on vaccinia virus expressing RABV glycoprotein (G) (V-RG) have been developed (7–9). Despite the fact that these vaccines generated a good protective immune response in many species, poor protective immunities were observed in dogs and skunks (10–12). The use of live attenuated RABVs also raised safety concerns about reversion to the pathogenic phenotype due to RNA genome mutation, residual virulence caused by vaccine overdose, or a change of target species (11, 13, 14). Vaccinia virus as a vaccine was reported to cause adverse local and systemic reactions in humans, and the vaccinia virus-vectored rabies vaccine (V-RG) was reported to have caused reactions in humans as well (15, 16). Although a modified vaccinia virus Ankara (MVA) vaccine expressing RABV G was thereafter developed as a safer substitute for the widely used V-RG, oral immunization of the recombinant MVA vaccine failed to induce anamnestic immune responses in dogs and raccoons with prior exposure (17). Therefore, there is a need to develop an efficacious and safe rabies vaccine for animals as well as for humans. For vaccinating wild animals, a rabies vaccine that can be orally administered is needed.

RABV, a member of the genus Lyssavirus of the Rhabdoviridae family, is an enveloped RNA virus possessing a single-stranded negative-sense genome with a bullet-shaped structure. The RNA genome encodes five structural genes in the following order: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and the viral RNA polymerase (L) (18). The N, P, and
Parainfluenza virus type 5 (PIV5) is a nonsegmented negative-strand RNA virus with a genome of about 15 kb in size and belongs to the *Paramyxovirus* family. PIV5 infects a broad spectrum of cell lines without a significant cytopathic effect (CPE), which supports the growth of PIV5 in continuous cell lines to obtain high titers, providing an economical means for mass production. Our previous result shows that a single-dose inoculation of recombinant PIV5 (rPIV5) containing the hemagglutinin (HA) gene of strain Udorn (rPIV5-H3) provides protection against influenza A virus challenge in mice (20). In addition, no association of PIV5 with human disease, no risk of integration of the virus genome into host DNA, and the stability of the negative-strand RNA virus genome over positive-sense RNA viruses suggest that PIV5 is a good vaccine vector and protein expression tool. Because kennel cough vaccines containing live PIV5 have been used in dogs for many years without raising safety concerns for animals or humans, we hypothesize that PIV5 expressing RABV G will be an effective vaccine for dogs and can be readily incorporated into existing dog vaccination programs. Developing a novel vaccine that is safe, is inexpensive to produce, and can be readily administered to animals will be important in controlling RABV in humans and animals. In this study, we tested the potential of PIV5 expressing RABV G as a novel rabies vaccine in mice.

**MATERIALS AND METHODS**

**Cells.** Cells of the cell lines BHK21, BSR-T7, and BSR, a cloned cell line derived from BHK21 cells (21), were maintained in Dulbecco’s modified Eagle medium (DMEM) with 10% tryptose phosphate broth, 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin. MDBK cells were grown in DMEM with 10% FBS. Mouse neuroblastoma (NA) cells were maintained in RPMI 1640 medium supplemented with 10% FBS. G418 was added to the medium of BSR-T7 cells to make a final concentration of 400 μg/ml. For virus infection, monolayers were washed with phosphate-buffered saline (PBS) and then inoculated with virus in DMEM plus 1% bovine serum albumin (BSA). The monolayers were then washed with PBS and incubated with DMEM containing 2% FBS at 37°C with 5% CO2.

**Viruses.** Wild-type PIV5 was described previously (22). To concentrate PIV5, the supernatant containing virus was loaded onto 20% sucrose and pelleted in a Thermo Scientific ultracentrifuge (type F40L-8/100 rotor) at 37,000 rpm for 1 h. The pellets were then resuspended in DMEM with 1% BSA and stored at −70°C.

The suckling-mouse-brain-adapted challenge virus standard 24 (CVS-24) rabies virus strain was propagated in suckling mice. Rabies vaccine strain LBNSE was derived from strain L16, which was reported previously (23), and grown in BSR cells. CVS-11 was propagated in NA cells. Fluorescein isothiocyanate (FITC)-conjugated antibody against the RV-N protein was purchased from Fujirebio Diagnostics, Inc. (Malvern, PA).

**Construction of virus infectious clone.** The PIV5 infectious clone plasmid pBH311 contains the green fluorescent protein (GFP) gene as an extra gene between the HN and L genes in the PIV5 genome, and the GFP is expressed (22). The full-length RABV G gene (1,575 nucleotides) was amplified by PCR from a plasmid containing the G gene from the RABV ERA strain. The primer sequences are as follows: RV-G1, 5’-AACAGGCGGCG CCAGGCGCCATGTTCTCAGGCTTCTCGTTTGTAC; RV-G2, 5’-AACAGGCGGCGGTCAAGTGCTGTCGTCACCCCACCT. The PCR fragment was inserted into the PIV5 infectious clone vector to generate a plasmid containing PIV5 G between HN and L as an extra gene, pPIV5-RV-G. The length of the genome of rPIV5 encoding RABV G (rPIV5-RV-G) was maintained as multiples of six. The details of plasmid construction and sequences are available on request.

**Rescue of rPIV5-RV-G.** To rescue rPIV5-RV-G, plasmid pPIV5-RV-G (3 μg), along with plasmids pCAGGS-PIV5-L (1.5 μg), pCAGGS-PIV5-NP (1 μg), and pCAGGS-PIV5-P (200 ng), were transfected into BSR-T7 cells. At 4 days posttransfection, supernatant containing rPIV5-RV-G was collected and plaque purified in BHK21 cells. Plaques (developing at 4 to 7 days postinfection [dpi]) were selected and further amplified in MDBK cells. RNA was extracted from the supernatant using a QIAamp viral RNA minikit, and reverse transcription (RT) was performed with random primers. The reverse transcription product was further amplified by PCR using specific primers binding the HN’ 3’ end or the L 5’ end. The primer sequences are as follows: 511-10096-F1, 5’-CAGAT GTGTTCCATTTTCGCTAGGGTGAC; 511-11764-R1, 5’-AGGTTGCT GCTATTTGGAATTTCCCAAG. The PCR products were sequenced.

**Growth curve and plaque assay.** MDBK cells in 6-well plates were infected with PIV5 or rPIV5-RV-G at a multiplicity of infection (MOI) of 0.01. The supernatants were collected at 0, 1, 2, 3, 4, and 5 dpi. For high-MOI infection, MDBK cells in 6-well plates were infected with PIV5 or rPIV5-RV-G at an MOI of 5, and the supernatants were collected at 0, 12, 24, 36, 48, and 60 h postinfection (p.i.). BHK21 cells in 6-well plates were infected with the virus stocks in serial dilution (1:10 to 1:106). After 48 h, the inoculating mixture was removed and replaced with 5 ml DMEM containing 2% FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 1% low-melting-point agarose. The plaques were counted at 5 to 6 dpi. Two replicates for each time point were used.

**Indirect immunofluorescence assay.** To detect expression of RABV G, rPIV5-RV-G-infected MDBK cells were examined by indirect immunofluorescence assay (IFA) as described before (24). Briefly, cells were fixed with 3.7% formaldehyde in PBS (pH 7.4) for 10 min and then permeabilized with 0.1% Triton X-100 plus 1% FBS in PBS for 30 min at room temperature. Fixed cells were incubated for 1 h with primary antibody (mouse anti-RABV-G antibody at a dilution of 1:200; Novus Biologicals, Inc.) at 37°C. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody (1:200 dilution; KPL, Inc.) was used as the secondary antibody.

**Western blotting.** rPIV5-RV-G-infected MDBK cells were lysed with whole-cell extraction buffer (WCEB: 50 mM Tris-HCl [pH 8], 280 mM NaCl, 0.5% NP-40, 0.2 mM EDTA, 2 mM EGTA, 10% glycerol) (23). The lysates were cleared by centrifugation at 4,000 rpm for 15 min, and the supernatants were mixed with the same volume of 2X SDS loading buffer (100 mM Tris-HCl [pH 6.8], 20% glycerol, 4% SDS, 200 mM dithiothreitol [DTT], 0.1% bromophenol blue), heated at 95°C for 5 min, and resolved by 10% SDS-PAGE. The proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane using an iblot dry blotting system (Invitrogen). The membrane was incubated with mouse anti-RABV-G antibody (1:2,000 dilution) or mouse anti-PIV5-V/P antibody (1:2,000 dilution, used for PIV5 infection control) (26), followed by incubation with goat anti-mouse secondary antibody labeled with horseradish peroxidase (HRP) at a dilution of 1:2,000. After washing, the PVDF membrane was incubated with ECL Advance substrate (GE Healthcare) and scanned using a Kodak Image Station 440 system.

**Purification of virus.** To determine whether RABV G is incorporated into recombinant PIV5 particles, viruses in the cleared supernatant were loaded onto 20% sucrose and pelleted in a Thermo Scientific ultracentrifuge (type F40L-8/100 rotor) at 37,000 rpm for 1 h. The pellets were then resuspended in DMEM with 1% BSA and stored at −70°C.

**Electron microscopy.** To examine incorporation of the RABV G protein in rPIV5-RV-G particles, purified PIV5 or rPIV5-RV-G virions were absorbed onto Parlodion-coated nickel grids for 30 s. Grids were then floated on a drop of Tris-buffered saline (TBS), pH 7.4, for 5 min, fol-
lowed by floating on drops of 3% ovalbumin in TBS for 1 h with RABV G-specific mouse monoclonal antibody diluted to 1:300 in 1% ovalbumin in TBS. After washing with TBS three times, samples were incubated for 1 h with goat anti-mouse IgG coupled to 10-nm gold particles diluted at 1:10 in 1% ovalbumin in TBS. Grids were again washed with TBS and then stained with 2% phosphotungstic acid, pH 6.6. The grids were then examined using a JEOl 1230 transmission electron microscope (JEOl, Tokyo, Japan).

Animal studies. Six- to 8-week-old female BALB/c mice were used in the animal studies. All animal experiments were performed following protocols approved by the Institutional Animal Care and Use Committee, University of Georgia. The mouse immunization is performed by the intranasal (i.n.), i.m., or oral route. For intranasal immunization, 6-week-old BALB/c mice were first anesthetized by intraperitoneal injection of tribromoethanol (Avertin; 180 to 250 mg/kg of body weight) and then inoculated intranasally by dropping 100 μl rPIV5-RV-G or PIV5 at different doses. PBS-treated mice served as controls. Three weeks later, the mice were boosted by use of the same dose of the first inoculation in the two-dose dose-response experiments. The mice were also immunized via oral administration of rPIV5-RV-G particles or intramuscular injection of 100 μl rPIV5-RV-G or PIV5 at different doses in the thigh muscle of the hind leg. As a rabies vaccine control, a group of mice was immunized by the i.m. route with 1 × 10^5 focus-forming units (FFU) of rabies vaccine strain LBNSE. The mice were bled from the tail prior to challenge for serological assessment.

The mouse challenge was carried out at 3 weeks postimmunization for the one-dose experiment or at 1 week postboosting for the two-dose experiment. Mice were infected with 50 50% lethal doses (LD50s) of RABV G. Our previous studies indicated that foreign genes (GFP and HA of the H3N2 subtype) inserted between the HN and L genes of PIV5 were expressed well in infected cells (20,22). The recombinant PIV5 expressing H3 as a live vaccine generated protective immunity against influenza A virus infection. To further explore the potential of PIV5 as a live vaccine, the feasibility of developing a recombinant rabies vaccine using the PIV5 vector was examined. The gene of the glycoprotein of the RABV EAR strain (RABV G) was inserted between the HN and L genes of PIV5 (Fig. 1A). The RABV G gene in the PIV5 genome was flanked with a gene start (GS) sequence, intergenic sequences (I), and a gene end (GE) sequence from the junction region of the NP and V/P genes, which gave rise to a high level of transcription (29). The virus was recovered, and the genome was confirmed by RT-PCR analysis and sequencing (data not shown).

Expression of the RABV G protein in rPIV5-RV-G-infected cells was detected by IFA. Cells infected with rPIV5-RV-G were stained by mouse monoclonal antibody against RABV G, while PIV5-infected cells were not stained (Fig. 1B). RABV G expression in rPIV5-RV-G-infected cells was further confirmed by Western blotting with mouse monoclonal antibody against RABV G (Fig. 1C). The size of recombinant RABV G (~65 kDa) is similar to that of the native RABV G (30).

To determine the effect of insertion of the RABV G gene on virus replication, multiple-step and single-step growth curves of PIV5 and rPIV5-RV-G were determined. In a multiple-step growth curve assay, MDBK cells were infected with 0.01 PFU per cell. A growth curve assay was performed. Mice were infected with 50 50% lethal doses (LD50s) of the one-dose experiment or at 1 week postboosting for the two-dose experiments. The mice were also immunized via oral administration of rPIV5-RV-G particles or intramuscular injection of 100 μl rPIV5-RV-G at different doses in the thigh muscle of the hind leg. As a rabies vaccine control, a group of mice was immunized by the i.m. route with 1 × 10^5 focus-forming units (FFU) of rabies vaccine strain LBNSE. The mice were bled from the tail prior to challenge for serological assessment.

The mouse challenge was carried out at 3 weeks postimmunization for the one-dose experiment or at 1 week postboosting for the two-dose experiment. Mice were infected with 50 50% lethal doses (LD50s) of the CVS-24 strain by the intracerebral (i.c.) route. Infected animals were observed daily for 22 days for symptoms of rabies virus infection.

**RESULTS**

**Generation and analysis of recombinant PIV5 expressing RABV G.** The gene of the glycoprotein of the RABV EAR strain (RABV G) was inserted between the HN and L genes of PIV5 (Fig. 1A). The RABV G gene in the PIV5 genome was flanked with a gene start (GS) sequence, intergenic sequences (I), and a gene end (GE) sequence from the junction region of the NP and V/P genes, which gave rise to a high level of transcription (29). The virus was recovered, and the genome was confirmed by RT-PCR analysis and sequencing (data not shown).

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cell (MOI, 0.01) of rPIV5 or rPIV5-RV-G, and supernatants were collected at 24-h intervals for up to 120 h. In a single-step growth curve assay, MDBK cells were infected with rPIV5 or rPIV5-RV-G at an MOI of 5, and supernatants were collected at 12-h intervals for up to 60 h. Viruses were quantified by plaque assay in BHK21 cells. As shown in Fig. 2A and B, both viruses had similar initial growth kinetics, though the growth rate for rPIV5-RV-G was a little lower than that of wild-type PIV5 at between 24 h and 96 h in multiple-step growth and between 12 h and 48 h in single-step growth. Both viruses reached similar maximum titers at about 120 h p.i. in multiple-step growth and 60 h p.i. in single-step growth. The result indicated that the introduction of the RABV G gene into the PIV5 genome as an extra expression unit did not significantly affect the growth of PIV5 in vitro.

Identification of RABV G incorporation into the rPIV5-RV-G particle. Since RABV G is an envelope protein, its incorporation into PIV5 particles was examined. PIV5 and rPIV5-RV-G were grown in MDBK cells and purified, and polypeptides were analyzed by SDS-PAGE and Western blotting. SDS-PAGE separated the viral proteins within the purified virions of PIV5 and rPIV5-RV-G into the major PIV5 structural proteins, including L, HN, NP, F, P, and M (Fig. 3). RABV G comigrated with the PIV5 NP on SDS gels, making it difficult to visualize RABV G by total protein staining of purified rPIV5-RV-G virions. To investigate the incorporation of G into rPIV5-RV-G, the virions of rPIV5-RV-G were analyzed using RABV G-specific antibody and examined using an electron microscope (EM). RABV G was detected in the virions of rPIV5-RV-G but not in those of PIV5 (Fig. 3B). The result indicated that RABV G is incorporated into the recombinant PIV5 particles. G incorporation and quantity were further confirmed using Western blot analysis of purified viruses with RABV G-specific antibody. An RABV G band was detected in the

FIG 2 Comparison of growth kinetics of PIV5 and rPIV5-RV-G in cells. (A) Multicycle growth assay. Multicycle growth curves of PIV5 and rPIV5-RV-G were performed in MDBK cells at an MOI of 0.01. Aliquots of supernatants from cell cultures were harvested at 24-h intervals until 120 h postinfection. (B) Single-cycle growth assay. Single-cycle growth curves of PIV5 and rPIV5-RV-G were performed in MDBK cells at an MOI of 5. Aliquots of supernatants from cell cultures were harvested at 12-h intervals for up to 60 h postinfection. The virus titers in the supernatants were determined by plaque assay in BHK21 cells. Values represent averages of the results from two independent experiments, and error bars show standard deviations.

FIG 3 Incorporation of RABV G in rPIV5-RV-G virions. (A) Composition of purified virions. The virus particles were purified through 10% to 80% (wt/vol) sucrose gradients. Viral proteins were analyzed by using 10% SDS-PAGE and were subjected to Western blot analysis with mouse antibody against RABV G. The position of the RABV G protein is indicated. (B) Detection of G in rPIV5-RV-G virions using EM. The purified virions were treated with anti-G and then secondary antibody labeled with gold particles. The samples were examined using EM. (C) Detection of G in rPIV5-RV-G using Western blot (WB) analysis. The RABV ERA strain stock was collected from cleared culture supernatants. The concentrated virions of rPIV5-RV-G were prepared through 20% sucrose, while the purified virions were made with 20% sucrose and 10% to 80% (wt/vol) sucrose gradients. Threefold serial dilutions were used for the RABV ERA strain stock and concentrated, and purified rPIV5-RV-G with known titers was obtained. The same volume of each diluted sample was loaded onto an SDS-polyacrylamide gel, which was followed by a Western blotting assay using antibody against RABV G. The RABV G levels in the panels for each virus were compared between ERA and concentrated rPIV5-RV-G or ERA and purified rPIV5-RV-G. The range of G in rPIV5-RV-G (concentrated or purified) was 12 to 15% of that in RABV with the same number of infectious particles.
rPIV5-RV-G virions, whereas no RABV G band was found in the PIV5 virions (Fig. 3C). Furthermore, the quantification showed that about 12 to 15% of G was incorporated into the rPIV5-RV-G virion compared to the amount in the RABV virion with the same number of infectious particles (Fig. 3C).

**Efficacies of rPIV5-RV-G in mice via intranasal inoculation.** To test whether rPIV5-RV-G can elicit enough protective immunity against a robust rabies virus challenge in the mouse model, a two-dose immunization regimen was performed. Because current vaccines containing live PIV5 are administered to dogs via the i.n. route, the efficacy of rPIV5-RV-G was first tested using i.n. vaccination. Four groups of mice were i.n. vaccinated with 10^3, 10^4, 10^5, or 10^6 PFU of rPIV5-RV-G. A control group received PBS by the i.n. route. Three weeks after prime vaccination, all mice were boosted with the same amount of initial inoculum. One week later, sera were collected and used for rabies virus VNA assays. Thereafter, the mice were challenged with 50 LD_{50}s of the rabies virus CVS-24 strain by the i.c. route. Titers of RABV neutralizing antibody (nAb) were determined following WHO guidelines, as described in Materials and Methods. As shown in Fig. 4A and B, the survival rate and the RABV nAb level displayed dose-dependent responses. The respective average VNA titers and survival rates (numbers of VNAs per survivor) for the groups vaccinated with 10^3, 10^4, 10^5, or 10^6 PFU of rPIV5-RV-G were 0.53 IU and 30%, 1.52 IU and 77.8%, 7.94 IU and 100%, and 62.96 IU and 100%, in contrast to 0 IU and 0% in the PBS-treated group. A value of 0.5 IU is considered to be the minimal level of protective antibody (31). The number of mice with VNAs higher than 0.5 IU in each group correlated strongly with its survival rate. For instance, in the group receiving 10^5 PFU of rPIV5-RV-G, 3 of 10 mice (30%) had VNAs higher than the minimal protective antibody level, and this group’s survival rate was 30%. All mice in the PBS group died within 9 days after challenge with RABV. The result showed that rPIV5-RV-G was able to elicit protective immune responses against rabies virus challenge in mice with a two-dose immunization schedule. The minimal effective two-dose regimen of rPIV5-RV-G for 100% protection was 10^6 PFU.

While the result of the two-dose regimen test demonstrated the immunogenic potential of rPIV5-RV-G against rabies virus challenge, we further examined whether rPIV5-RV-G can be effective with a one-dose vaccination in mice. Three groups of mice were i.n. vaccinated with 10^5, 10^6, or 10^7 PFU of rPIV5-RV-G. A control group received 10^7 PFU of PIV5 by the i.n. route. Three weeks after immunization, the mice were challenged with 50 LD_{50}s of the rabies virus CVS-24 strain by the i.c. route. As shown in Fig. 5A and B, the groups of mice i.n. immunized with 10^5, 10^6, or 10^7 PFU of rPIV5-RV-G displayed dose-dependent increases in average VNA titers and had survival rates of 77.8%, 100%, and 100%, respectively. Thus, a single dose of 10^6 PFU of rPIV5-RV-G with i.n. vaccination was the minimal dose for 100% protection against rabies virus challenge.

**Efficacies of rPIV5-RV-G in mice via i.m. inoculation.** In some circumstances, i.m. vaccination may be preferred. Thus, the efficacy of i.m. immunization was examined. At the same time as the i.n. inoculation, three groups of mice were injected with 10^6, 10^7, or 10^8 PFU of rPIV5-RV-G via the i.m. route. At 3 weeks postimmunization, the mice were challenged with 50 LD_{50}s of the rabies virus CVS-24 strain by the i.c. route. As shown in Fig. 5A and B, the groups vaccinated with 10^6, 10^7, or 10^8 PFU via the i.m. route displayed dose-dependent increases in average VNA titers and showed survival rates of 60%, 70%, and 90%, respectively.

**Efficacies of rPIV5-RV-G in mice via oral inoculation.** Effective vaccination via the oral route will be critical for successful vaccination of wild animals. To determine the efficacy of the vaccine candidate via oral immunization, three groups of mice were...
vaccinated with 10^6 PFU of rPIV5-RV-G via the i.n. route, 10^8 PFU of rPIV5-RV-G via the i.m. route, or 10^8 PFU of rPIV5-RV-G via the oral route. Two negative-control groups received inoculation with 10^6 PFU of PIV5 or PBS via the i.n. route. In addition, a control group of mice was vaccinated with 10^7 FFU of the recombinant LBNSE (rLBNSE) strain, an attenuated rabies vaccine, via the i.m. route. Sera were collected for RV VNA assays 3 weeks after vaccination, and the mice were challenged with 50 LD_{50} of the rabies virus CVS-24 strain by the i.c. route. As shown in Fig. 6B, all mice receiving 10^6 PFU of rPIV5-RV-G via the i.n. route and 10^8 PFU of rPIV5-RV-G via the i.m. route survived the challenge. The mice vaccinated via the oral route with 10^8 PFU of rPIV5-RV-G had a 50% survival rate, while 60% of the rLBNSE strain-vaccinated mice survived. All mice in either the PIV5 or the PBS group were dead within 9 days after challenge. The highest average level of VNA was detected in the group that received 10^6 PFU of rPIV5-

FIG 5 Efficacy of one-dose immunization of rPIV5-RV-G against rabies virus challenge in mice. Groups of mice (n = 10, each group) were immunized intranasally with 10-fold-diluted rPIV5-RV-G (10^5 PFU to 10^7 PFU) or vaccinated intramuscularly with rPIV5-RV-G (10^6 PFU to 10^8 PFU). Control mice were inoculated with 10^6 PFU of PIV5. Three weeks later, all mice in each group were challenged with 50 LD_{50} of CVS-24 strain by the i.c. route. (A) VNA test. Serum samples were collected before challenge and used for measuring the VNA titer against rabies virus by RFFIT. Bars representing the average VNA titer of each group and the values for average VNA are shown. VNA titers between 10^6 and 10^7 PFU delivered i.n. and 10^5 and 10^6 PFU delivered i.m. were statistically significant (P = 0.018 and 0.008, respectively). VNA titers between 10^5 and 10^6 PFU delivered i.n. and 10^6 and 10^7 PFU delivered i.m. were not statistically different (P = 0.11 and 0.402, respectively). (B) Survival rates. Infected animals were observed daily for 22 days for clinical signs of rabies.

FIG 6 Efficacy of oral immunization of rPIV5-RV-G against rabies virus challenge in mice. Groups of mice (n = 10, each group) were immunized intranasally with one dose of 10^6 PFU of rPIV5-RV-G or vaccinated with 10^8 PFU of rPIV5-RV-G by the i.m. or oral route. As a positive control, a group of mice was immunized by the i.m. route with 1 x 10^7 FFU of rabies vaccine strain LBNSE. Control mice were inoculated with 10^6 PFU of PIV5 or with PBS. Three weeks later, all mice in each group were challenged with 50 LD_{50} of the CVS-24 strain by the i.c. route. (A) VNA test. Serum samples were collected before challenge and used for measuring the VNA titer against rabies virus by RFFIT. Bars representing the average VNA titer of each group and the values for average VNA are shown. VNA titers between the i.n. (10^6 PFU) and i.m. (10^6 PFU) routes were not statistically different (P = 0.66, calculated using Student’s t test). VNA titers between the oral and i.n. or i.m. route were statistically significantly different (P = 0.03 and 0.003, respectively). (B) Survival rates. Infected animals were observed daily for 22 days for clinical signs of rabies.
RV-G via the i.n. route (Fig. 6A). Overall, the average level of VNA correlated positively with the survival rate, as expected.

DISCUSSION

During the past decade, a number of recombinant rabies vaccine candidates based on live attenuated RABV or recombinant viruses expressing RABV G (such as V-RG) have been developed as potential alternatives to current rabies vaccines (17, 29, 32–35). While some of the vaccine candidates generated protective immunity when administered via the i.m. route, data on the efficacy of oral immunization with these candidates are lacking. Of those reported, oral immunization with canine adenovirus carrying the rabies virus G gene did not confer protection against rabies virus infection in mice (32). As a safer alternative to V-RG, the recombinant MVA vaccine expressing a rabies virus glycoprotein gene was generated and tested in mice, and the result showed that protection was observed only in mice vaccinated with a dose as high as 106 PFU by a peripheral route (17). In this work, to demonstrate infection in mice (32). As a safer alternative to V-RG, the recombinant MVA vaccine expressing a rabies virus glycoprotein gene was generated and tested in mice, and the result showed that protection was observed only in mice vaccinated with a dose as high as 106 PFU by a peripheral route (17). In this work, to demonstrate the proof of principle, we inserted the G gene of RABV between the HN and L genes of PIV5 and found that this vaccine candidate was effective via oral immunization as well as i.n. and i.m. immunization. To our knowledge, this is the first demonstration of oral immunization efficacy in mice for a rabies virus vaccine using paramyxovirus as a vaccine vector. The results also suggest that PIV5 can be used as a vector for developing vaccines for which oral delivery is essential. Further development of the PIV5-based rabies vaccine may lead to a novel rabies vaccine for humans and for animals.

We tested three immunization routes in mice. Of those, i.n. inoculation gave the best immune responses and protection, demonstrating that rPIV5-RV-G can elicit a protective immune response against rabies. i.n. immunization has been used in many pet dogs in the United States for many years, such as for kennel cough vaccination. The fact that PIV5-RV-G was effective via i.n. immunization suggests that it is possible to incorporate it into the existing canine vaccination program. We have found that the i.m. route of immunization was more effective at higher doses. In this work, we detected incorporation of RABV G into rPIV5-RV-G virions (Fig. 3). It has been reported before that there are about 1,200 RABV G proteins (400 trimers) in RABV virions (36, 37). Thus, there were about 150 to 180 G proteins in rPIV5-RV-G. We speculate that the higher that the dose of PIV5-RV-G was, the more G protein was injected in the i.n. immunization. In our recent work, highly efficacious immunization with a PIV5-based vaccine via the i.m. route does require virus to be live, since the inactivated PIV5-based vaccine provides only partial immunity when it is administered via the i.m. route (38). While a single-dose inoculation provided immunity against lethal rabies virus challenge in mice, a boost further enhanced the efficacy of rPIV5-RV-G: prime-boost with 105 PFU of rPIV5-RV-G provided 100% protection, whereas 77% protection was afforded by a single dose of 105 PFU via the i.n. route of inoculation. Average VNA titers increased from 2.76 IU with one dose of 105 PFU to 7.94 IU with the two-dose vaccination. Most remarkably, VNAs in mice were increased from 4.73 IU with one dose to 62.96 IU with two doses of 105 PFU of pPIV5-RV-G via i.n. inoculation. The anamnestic immune response was elicited in mice vaccinated with pPIV5-RV-G, suggesting that prior exposure to PIV5 did not prevent a robust immune response to PIV5-vectored antigen. One of the concerns over using an rPIV5-RV-G vaccine is whether preexisting anti-PIV5 immunity will negatively influence the efficacy of the PIV5-vectored vaccine. The robust immune responses from the boost with live vaccine suggest that preexisting immunity to the viral vector did not affect the efficacy of the PIV5-based vaccine. Recently, we have found that dogs with neutralizing antibodies against PIV5 generated a protective immune response against influenza virus after immunization with PIV5 expressing HA of influenza A virus, demonstrating that a PIV5-based vaccine is effective in dogs with prior exposure (39). The use of rabies vaccines, especially live attenuated ones, in newborn dogs is limited due to maternal antirabies antibody, which can last as long as 6 months. The PIV5-based rabies vaccine provides an alternative that can effectively be used to vaccinate newborn dogs, further demonstrating the benefit of using a PIV5-based rabies vaccine for dogs.

While i.n. immunization provided the best protection for rPIV5-RV-G, to vaccinate stray dogs or wild animals, oral immunization will be the best approach. Oral vaccine has advantages over traditional vaccines, such as its ease of use, compatibility with mass immunization campaigns, and ability to reach hard-to-reach species (40). Although only half of the mice in our study were protected with one-dose vaccination, the result is encouraging. It is comparable to the rate achieved with the current live rabies vaccine. The protection mechanism of oral vaccination is poorly understood. The VNA titer of serum from peripheral blood from mice vaccinated orally indicated a systemic immune response. The range of VNA titers from the oral inoculation was from 0.1 IU to 3.8 IU, with the average titer being 1.5 IU. We speculate that PIV5 was able to deliver antigen to mucosal cells by the oral route, which resulted in specific immune responses, including a systemic response to the PIV5-vectored antigen. It is possible to further increase the protection efficacy of oral inoculation by using a prime-boost regimen. In addition, modification of the PIV5 vector may increase the efficacy of the PIV5-vectored rabies vaccine. In our recent work, we have found that the insertion site of a foreign gene within PIV5 affects the immunogenicity of the PIV5-based vaccine (41). For instance, insertion of HA of H5N1 between SH and HN within PIV5 results in a vaccine candidate that generates better immunity against H5N1 challenge than a vaccine with the insertion of HA of H5N1 between HN and L within PIV5 in mice (41). We can further improve the efficacy of the PIV5-based vaccine for oral immunization by inserting the G gene in different places within the PIV5 genome. In addition, expression of additional rabies virus antigens may enhance the potency of the PIV5-based vaccine. It has been reported before that the rabies RNP can be protective (42). For example, expression of one of the rabies virus proteins N, P, or L together with G using PIV5 may enhance immune protection efficacy.

The fact that the PIV5-based rabies vaccine generated a robust immune response that protected against a lethal challenge from rabies virus infection demonstrates the potential of PIV5 as a vaccine that may be used to control rabies virus infection in dogs as well as its potential to be a vector for other infectious diseases in dogs and other animals as well as in humans. Because live PIV5 has been used in dogs for many years without safety concerns for dogs and humans, incorporating a PIV5-based vaccine into existing vaccination programs for dogs should be feasible.

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