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Oral immunization of mice with recombinant rabies vaccine strain (ERAG3G) induces complete protection

Dong-Kun Yang[1], Ha-Hyun Kim[1], Sung-Suk Choi[1], Jong-Taek Kim[2], Woong-Ho Jeong[3], Jae-Young Song[1]

[1]Viral Disease Division, Animal and Plant Quarantine Agency, MAFRA, Anyang; [2]College of Veterinary Medicine, Kangwon National University, Chuncheon; [3]Gangwon-do Veterinary Service Laboratory, Chuncheon, Korea

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Corresponding author: Dong-Kun Yang, PhD, DVM
Animal and Plant Quarantine Agency, 175 Anyang-ro, Anyang 430-757, Korea
Tel: +82-31-467-1783, Fax: +82-31-467-1797
E-mail: yangdk@korea.kr

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Purpose: New rabies vaccine bait for both pets and raccoon dogs residing in Korea is needed to eradicate rabies infection among animals. In this study, we constructed a recombinant rabies virus (RABV), the ERAG3G strain, using a reverse genetics system. Then we investigated the efficacy of this strain in mice after oral administration and the safety of this strain in cats after intramuscular administration.

Materials and Methods: The ERAG3G strain was rescued in BHK/T7-9 cells using the full-length genome mutated at the amino acid position 333 of the glycoprotein gene of RABV and helper plasmids. Four-week-old mice underwent one or two oral administrations of the ERAG3G strain and were challenged with the highly virulent RABV strain CVSN2c 14 days after the second administration. Clinical symptoms were observed and body weights were measured every day after the challenge.

Results: All mice showed complete protection against virulent RABV. In addition, cats intramuscularly inoculated with the ERAG3G strain showed high antibody titers ranging from 2.62 to 23.9 IU/mL at 28-day postinoculation.

Conclusion: The oral immunization of the ERAG3G strain plays an important role in conferring complete protection in mice, and intramuscular inoculation of the ERAG3G strain induces the formation of anti-rabies neutralizing antibody in cats.

Keywords: Recombinant rabies virus, Reverse genetics, Mouse, Animals

Introduction

Rabies, one of the most fatal zoonotic diseases, is caused by infection with the rabies virus (RABV) of the genus *Lyssavirus* (family *Rhabdoviridae*, order *Monogegavirales*). Small- to medium-sized carnivores serve as vectors in the transmission of RABV worldwide. Stray dogs are the major reservoir of RABV in most developing countries, whereas wild animals are largely responsible for the dissemination of rabies in many developed countries [1]. In Korea, 33 human rabies cases and 756 animal rabies cases have been reported since 1970 according to information provided by the KAHIS program (http://www.kahis.go.kr) of the Animal and Plant Quarantine Agency (QIA). Although urban rabies infection induced by dog bites has not been reported in Korea since 1993, sylvatic rabies, which is spread by two wild carnivores (raccoon dogs [*Nyctereutes procynoide koresis*] and badgers [*Meles meles*]), is still identified in some counties of Gyeonggi and Gangwon provinces. Most animal rabies cases are associated with the bite of a raccoon dog [2].

To prevent transmission of rabies from wild animals to domestic and companion animals, many countries have taken preventive measures such as distribution of vaccine baits. Several strains of rabies vaccine baits have been developed and applied to wild animals worldwide. The first oral rabies vaccine (ORV) strain is SAG2 (Street Alabama Dufferin [SAD]-avirulent-gif), which was mutated from the SAD Bern strain by utilizing antiglycoprotein monoclonal antibodies. The SAG2 strain produces no clinical illness in experiment animals, and all vaccinated dogs and raccoons show protection from challenge with virulent RABV [3,4]. The second ORV strain is a recombinant adenovirus strain from which both the E1 and E3 gene loci are deleted. This recombinant adenovirus expressing the rabies glycoprotein can induce the formation of neutralizing antibody in dogs, skunks, and raccoons [5]. The third ORV strain is a canarypox-rabies glycoprotein recombinant vaccine, which has also been found to be effective for animals [6]. The fourth ORV strain is a recombinant pseudorabies virus that expresses RABV glycoprotein and has shown safety and immunogenicity in dogs [7]. Finally, the last ORV strain is a vaccinia-rabies virus glycoprotein recombinant virus (V-RG) that expresses the glycoprotein of the Evelyn-Rokitnicki-Abelseth (ERA) strain. Use of V-RG has reportedly achieved containment or elimination of wild animal rabies in the United States, Canada, and several other countries [8].

The V-RG vaccine has also been distributed in areas at high risk of rabies in Korea since 2000 [9]. Because the V-RG vaccine has helped to prevent spread of wild animal RABV infection in both European countries and the Americas, ORV with V-RG has led to a gradual reduction in the number of rabies cases in Korea [2,9]. Nevertheless, the commercial V-RG vaccine contains a high titer of a recombinant vaccinia virus and may cause adverse effects in nontarget animals, including humans. Human vaccinia infection by V-RG vaccine bait was reported in a woman with a chronic skin condition in the United States [10]. In addition, V-RG induces protective immunity only in foxes and raccoons, not in dogs or skunks [11], indicating that there is a need for a safe and potent ORV vaccine strain for ownerless and stray dogs and wild animals other than foxes and raccoons.

Generally, street RABVs are neuroinvasive and pathogenic in animals, but attenuated strains have a limited ability to invade the central nervous system and can trigger a strong immune response [12]. Previous studies have elucidated upregulation of cytokines and receptors related to the innate immune response in mice following infection with a fixed RABV strain [13,14]. On the other hand, it is also known that raccoon exposure to street RABV does not always result in clinical disease, and some raccoons survive RABV challenge because of interindividual differences in innate immune responses [15]. Therefore, new alternative rabies vaccine baits applicable to dogs and raccoon dogs residing in Korea are needed to more effectively eradicate animal rabies.

In this study, we investigated the efficacy of a recombinant RABV, the ERAG3G strain constructed with reverse genetic system in mice after oral administration and the safety and immunogenicity of this strain in cats after intramuscular administration.

Materials and Methods

Cells and viruses

The BHK/T7-9 cells, a cloned cell line derived from BHK-21 cell [16,17] were maintained in Dulbecco's modified Eagle medium (DMEM) with 10% of tryptose phosphate broth, 5% of heat inactivated fetal bovine serum (FBS) and antibiotics (100 IU mL penicillin, 10 μg/mL streptomycin, and 0.25 μg/mL amphotericin B). Hygromycin was added to the medium of BHK/T7-9 cells to make a final concentration of 600 ng/mL. Murine neuroblastoma (NG108-15) cells for propagating recombinant RABV were maintained in DMEM supplemented with 5% of FBS and were put at 37°C in 5% CO_2 incubator. The virulent RABV, challenge virus standard (CVSN2c) was used for measuring efficacy in mice. CVS-11 strain was propagated in BHK-21 cells and used for virus neutralizing antibody test.

Construction of recombinant RAVA

Full-length cDNA modified into Arg-to-Glu mutation at position 333 amino acid of glycoprotein gene of the ERA strain was cloned into the pUC19 vector. The N, P, and L genes obtained from ERA strain were also cloned into the same vector. After carrying out transfection, a recombinant RAVA named as ERAG3G strain was recovered in BHK/T7-9 cells [18]. After second passage of constructed recombinant RABV, the titration of the ERAG3G was also done by indirect fluorescent assay (IFA) and the viral titers were determined by 50% fluorescent assay infectious dose per milliliter ($FAID_{50}$/mL). In addition, the authenticity of G gene of the ERAG3G stain was confirmed by direct sequencing of reverse transcription-polymerase chain reaction fragments.

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Efficacy of ERAG3G strain in mice

Four-week-old BALB/c mice were divided into four groups composing four female mice. Group 1 was administrated with the ERAG3G strain per oral once, group 2 with the ERAG3G strain twice every two weeks, group 3 remained as control 1, but was challenged with CVSN2c strain, and group 4 remained as control 2 without any treatment. Each mouse belonging to groups 1 and 2 was administrated with 200 μL of the ERAG3G virus ($10^{8.0}$ $FAID_{50}$/mL) via oral route. The clinical symptoms were observed every day after administration. To check efficacy of the ERAG3G strain, all mice except group 4 were challenged by intramuscular injection with a lethal dose of 100 μL of 25 LD_{50}/0.1 mL (CVSN2c strain) into the right leg. Body weight of each mouse was measured after challenge for 9 days and survival of mice was observed for 12 days after challenge.

Safety and immunogenicity of ERAG3G strain in cats

The 51-year-old cats showing various antibody levels against RABV were inoculated with the ERAG3G virus (1 mL, $10^{8.0}$ $FAID_{50}$/mL) via intramuscular route. All cats were monitored daily for adverse effects such as anorexia, prostration, anxiety, agitation, aggression, and paralysis. Following inoculation at 4 weeks, blood was collected from all cats to measure neutralizing antibody against RABV.

Serological assay

Virus neutralizing (VN) antibody titer against RABV was determined by the fluorescent antibody virus neutralization test [19]. In short, a positive reference serum of world Health Organization (WHO) adjusted to 0.5 IU/mL was used as a positive control. Each serum sample as well as the positive and negative controls were distributed in four consecutive wells, and then serially diluted in three-fold. The RABV (CVS-11 strain) containing around 100 $FAID_{50}$/50 μL was then added to each well. After 60 minutes of incubation at 37°C, a volume of 50 μL of BHK-21 cells suspension containing 4×10^5 cells/mL was added to each well and the microplates were incubated for 72 hours in a humidified incubator with 5% CO_2 at 37°C. The microplates were fixed in cold acetone (–20°C) for 20 minutes. After three successive washings with phosphate buffered saline (PBS) (pH 7.2), the microplates were reacted with specific monoclonal antibody against rabies for 45 minutes at 37°C, and then stained with fluorescence isothiocyanate conjugated goat-anti mouse IgG+ IgM. After rinsing with PBS, the microplates were air-dried and were examined at 200× using a fluorescent microscope (Nikon, Tokyo, Japan). The titers of serum samples were expressed in International Units per milliliter (IU/mL) by comparing results obtained with those of the positive standard.

Results

Construction of recombinant RABV

Using the full-length genome mutated in the glycoprotein gene of the ERA strain and three helper plasmids (N, P, and L), a recombinant RABV designated as the ERAG3G strain was successfully constructed in BHK/T7-9 cells. The cytopa-

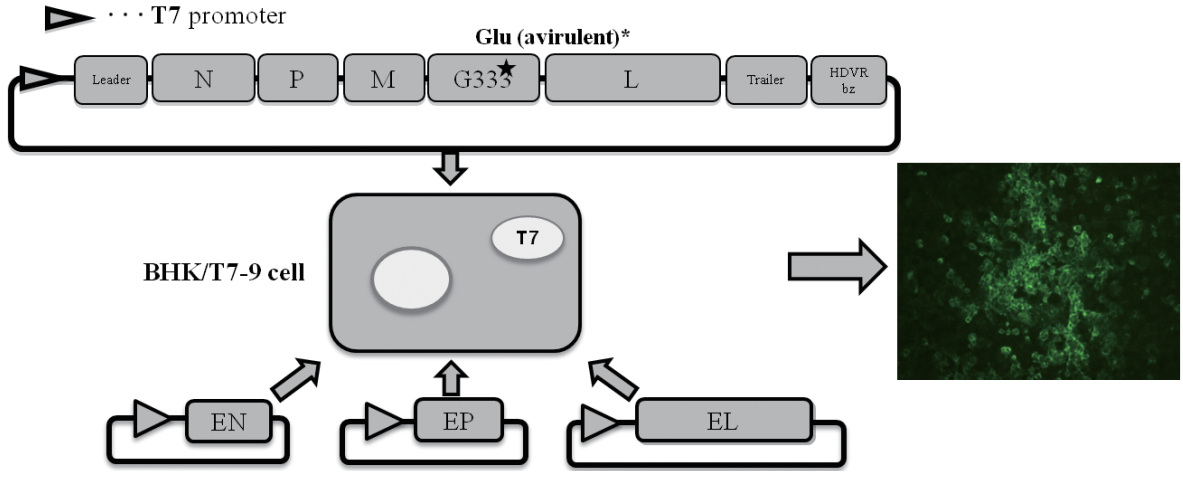

Fig. 1. The ERAG3G strain was constructed by reverse genetic system. The full-length cDNA plasmid and three helper plasmids (EN, EP, and EL plasmid) were co-transfected into BHK/T7-9 cells producing RNA polymerase, respectively. The viral titer of ERAG3G strain propagated in NG108-15 cells reached $10^{8.0}$ $FAID_{50}$/mL.

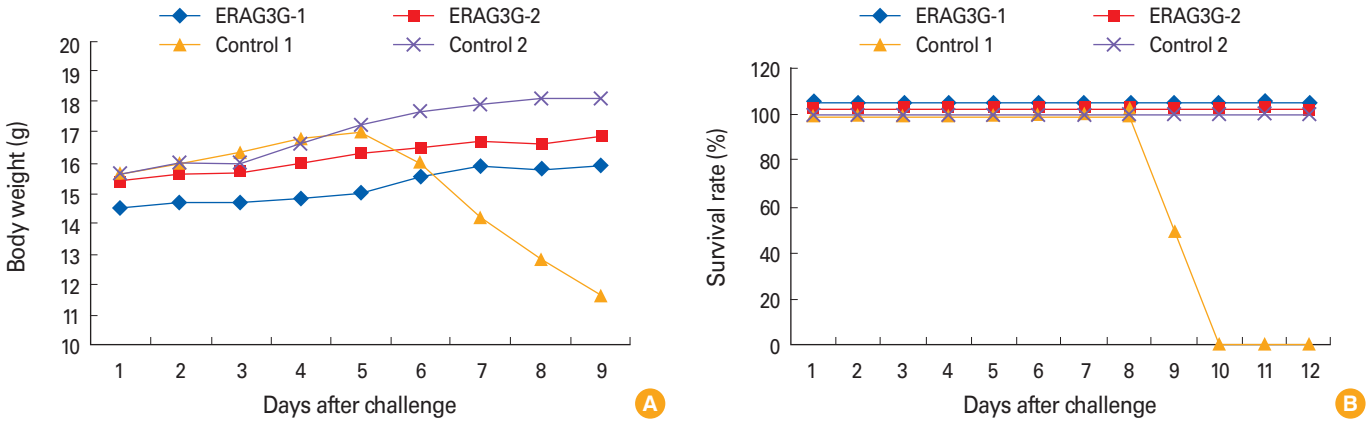

Fig. 2. Change of body weight (A) and survival rate (B) in 4-week-old mice immunized with ERAG3G strains via oral route and challenged with highly pathogenic rabies virus strain (CVSN2c).

thic effect, which was characterized by rounding and shrinking, was detected after a 3-day incubation period in BHK/T7-9 cells inoculated with the ERAG3G strain. The BHK/T7-9 cells inoculated with the ERAG3G strain were fixed with cold acetone and reacted with specific monoclonal antibody against the nucleocapsid protein of RABV. RABV-specific fluorescence appeared in the cytoplasm of the infected cells (Fig. 1). The ERAG3G strain was propagated well in NG108-15 cells and exhibited a titer of $10^{8.0}$ $FAID_{50}$/mL.

Efficacy of ERAG3G strain in mice

The body weights of 4-week-old mice orally inoculated with the ERAG3G strain were measured every day after challenge. As shown in Fig. 2A, the average body weight of the mice that underwent one or two oral administrations of ERAG3G strain increased moderately for 9 days after the challenge. On the other hand, the average body weight of the control 1 mice began to drop more quickly from day 5 after the challenge (Fig. 2A), but that of the control 2 mice increased for 12 days after the challenge with no clinical symptoms of rabies.

The 4-week-old mice that underwent oral administration of the ERAG3G strain and the control 1 mice were challenged with the highly virulent RABV strain CVSN2c 14 days after the second administration. Survival of all mice, including those in the control 2 group, was observed every day after the challenge. As shown in Fig. 2B, all mice in groups 1, 2, and control 2 were alive with no clinical signs of rabies for 12 days, and the survival rates did not change during the observation period. There was no difference in the survival rate between mice that underwent one versus two administrations of the ERAG3G strain. All control 1 mice died of rabies 10 days after the challenge.

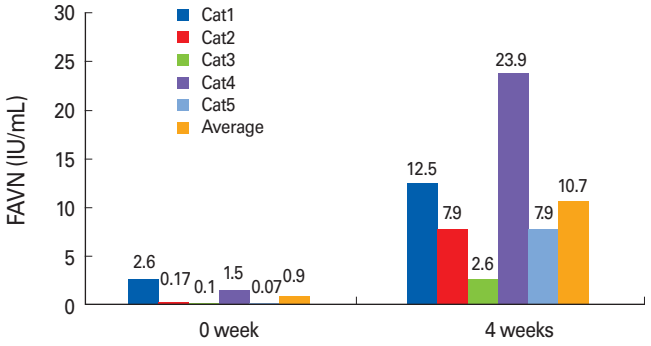

Fig. 3. The neutralizing antibody titer of cats inoculated with ERAG3G strain via intramuscular route. All cats designated as Cat1 to Cat5 immunized with the ERAG3G strain induced high neutralizing antibody titer (2.6-23.9 IU/mL) > 0.5 IU/mL against rabies virus. FAVN, fluorescent antibody virus neutralisation.

Immunogenicity of the ERAG3G strain in cats

All cats inoculated with the ERAG3G strain showed seroconversion 28 days after inoculation. Vaccinated cats exhibited no clinical signs of rabies during the observation period. All cats intramuscularly inoculated with the ERAG3G strain developed a rabies VN titer of 2.62 to 23.9 IU/mL (geometric mean, 10.7 IU/mL) 28 days after inoculation (Fig. 3).

Discussion

Many human pathogens have originated in animals, and 75% of emerging animal diseases have been transmitted to humans [20]. In particular, human rabies is closely related to animal rabies outbreaks, suggesting that the "One Health" concept of improving the health of all species through the collaboration of human and veterinary medicine should be introduced to rabies. Rabies has occurred in most Asian coun-

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tries with the exception of Brunei, Hong Kong, Japan, Malaysia, and Singapore, and vector hosts differ depending on the countries [21]. In South Asian countries such as Vietnam, India, and Thailand, stray dogs are responsible for transmitting the disease to livestock and humans; in China and Taiwan, wild animals such as Chinese ferret badgers play a key role transmitting the disease; and in Korea, raccoon dogs are considered to be a major vector species [2,22]. Control measures including strict, massive vaccination of pets and domestic animals raised within rabies risk regions as well as management of ownerless dogs and cats have been carried out to eliminate animal rabies. Several strategies such as the ORV program, trap-vaccinate-release program, point infection control, and reduction population of vector animals have been applied to many provinces of affected countries [23]. These programs have contributed to the elimination of rabies in several countries such as Finland, the Netherlands, Italy, and Canada [24].

Reverse genetics techniques for recovering recombinant RABV from cloned cDNA have been reported with the HEP-Flury, Ni-CE, and SAD-B19 strains [25-27]. Recovery of recombinant virus from BHK/T7-9 cells transfected with full-length cDNA and three helper plasmids for compatible partners among the ERA genome and the optimization of helper plasmids at the proper plasmid concentrations are difficult [16]. In this study, full-length cDNA mutated at position 333 (Arg-to-Glu) of the glycoprotein gene of the ERA strain and three helper plasmids based on the sequence of the ERA strain were prepared. After transfection, recombinant ERAG3G virus was rescued in BHK/T7-9 cells. Thus, the optimal transfection condition used in this study appears to be suitable for recovery of recombinant RABV in BHK/T7-9 cells. The ERAG3G strain was confirmed by IFA testing using specific monoclonal antibodies, indicating that the ERAG3G strain can be propagated well in both NG108-15 and BHK/T7-9 cells.

The amino acids arginine (Arg) or lysine (Lys) at position 333 of the glycoprotein of fixed RABV is responsible for virulence in warm-blooded animals [28]. To reduce pathogenicity, another amino acid such as glutamine, leucine, or glycine was substituted for Arg in the ORV strains by selecting an escape mutant or using a reverse genetics system [3,25]. Then it was proven that the SAG2 or rHEP^{333}R strain containing a glutamine at position 333 exhibited no pathogenicity and induced efficacy in several different animal species [3,25]. In our previous study, we showed that intramuscular or intracranial administration of the ERAG3G strain to 4- or 6-week-old mice was safe and provided complete protection against challenge [18]. In this study, mice orally inoculated with ERAG3G containing $10^{8.0}$ $FAID_{50}$/mL once or twice were observed for 28 days without displaying any clinical signs. The mean body weight of the mice did not decrease after inoculation, indicating that the ERAG3G strain is not pathogenic to 4-week-old mice. Moreover, in the efficacy test, 4-week-old mice administrated with the ERAG3G strain once or twice were challenged with 25 LD_{50}/0.03 mL intracranially, and all mice survived for 12 days. The mice exhibited no clinical signs or reductions in body weight, indicating that one or two oral immunizations with ERAG3G strain conferred complete protection against the RABV challenge.

Because carnivores appear to be the primary hosts of RABV, it is necessary to evaluate the safety and immunogenicity of the ORV candidate in major target animals of RABV. Oral immunization with modified live RABV strains (ERA and SAD Bern) was performed to prevent animal rabies in wildlife in the late 1960s, but the ERA and SAD Bern strains were found to have a degree of residual pathogenicity in wild animals, including cats [8,29]. Therefore, it is necessary to investigate the pathogenicity of the ERAG3G strain in carnivores. In our previous study, intramuscular administration with the ERAG3G strain was safe and induced high neutralizing antibody titers against RABV in dogs [16].

In this study, we also demonstrated that all cats inoculated with the ERAG3G strain containing a titer of $10^{8.0}$ $FAID_{50}$/mL developed no behavioral adverse effects. All immunized cats had high neutralizing antibody titers of 2.62 to 23.9 IU/mL 4-week postinoculation. Thus, it is assumed that cats inoculated with the ERAG3G strain have protection against challenge with virulent RABV, which provides a basis for use of a live RABV strain. However, further study concerning the use of ORV in accordance with the National Standard Assay for Veterinary Biologic Products is needed in dogs and raccoon dogs. In addition, the full-length RABV cDNA containing several restriction enzyme sites may be useful for recovering recombinant RABVs expressing other foreign genes.

In conclusion, a single oral administration of the ERAG3G strain induced complete protection against virulent RABV in mice. Intramuscular immunization with the ERAG3G strain in cats was safe and induced high neutralizing antibody titers. Therefore, the ERAG3G strain may be useful as an oral vaccine bait for both domestic and wild animals.

ORCID

Dong-Kun Yang *http://orcid.org/0000-0001-5765-3043*
Ha-Hyun Kim *http://orcid.org/0000-0001-6473-0035*
Sung-Suk Choi *http://orcid.org/0000-0001-5124-9736*
Jong-Tack Kim *http://orcid.org/0000-0002-6388-550X*
Woong-Ho Jeong *http://orcid.org/000-0003-1776-8297*
Jae-Young Song *http://orcid.org/0000-0002-9554-9252*

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