Safety and Immunogenicity of a Recombinant Rabies Virus Strain (ERAG3G) in Korean Raccoon Dogs

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A new alternative rabies bait vaccine strain named ERAG3G, which is applicable to wild animals, was developed to eliminate rabies in South Korea. In this study, the safety and immunogenicity of the strain was evaluated in Korean raccoon dogs. The ERAG3G was propagated in BHK/T7-9 cells. Korean raccoon dogs were administered ERAG3G (1 ml, 10^8.0 FAID50/ml) orally or intramuscularly to evaluate its safety and immunogenicity. The raccoon dogs were observed for 70 days after administration, and immunogenicity was measured using a fluorescent antibody virus neutralization test. The ERAG3G strain was not pathogenic to Korean raccoon dogs immunized via the intramuscular or oral route. Raccoon dogs administered the candidate vaccine via the oral route developed high virus neutralizing antibody (VNA) titers ranging from 13.7 to 41.6 IU/ml 70 days post administration. Raccoon dogs inoculated intramuscularly with the ERAG3G strain developed moderate VNA titers ranging from 0.5 to 13.7 IU/ml. These findings suggest that the ERAG3G strain is safe and induces a protective immune response in raccoon dogs.

Key Words: Recombinant rabies virus, Immunity, Raccoon dogs

INTRODUCTION

Rabies causes about 55,000 human deaths per year, indicating that it is a dangerous public health problem and one of the most important zoonoses in the world (1). Most human rabies cases are related to a bite from a rabid dog, and dogs are the main vector in many countries, including those in Asia. However, wild animals such as raccoons, raccoon dogs, badger, foxes, and mongoose have been associated with the rabies virus (RABV) circulating in Eastern Europe and Eastern North America since the late 1990s. Raccoon dogs (Nyctereutes procyonoides koreensis) remain the main wildlife vector in Korea (2). The estimated Korean raccoon dog and stray dog population is 260,000, and the wild animal bite incidence, including bites from raccoon dogs, has been 4.5% among 2,310 bites reported since 2011. Raccoon dog rabies has affected high-risk rabies regions in two Korean provinces, and the epizootic disease has recently extended into southern areas of the Han River (3). Although the eradication of sylvatic rabies by a trap-vaccination-release program is optimum, wild animals, in-
including the raccoon dog, are difficult to trap (4). In addition, most raccoon dogs are too aggressive to administer a parenteral vaccination; intramuscular vaccination is not usually possible, as they are stray, free-ranging, and fierce dogs.

Oral rabies vaccination (ORV) with a modified live virus via the oral route has been successful in field trials to prevent rabies in wild animals and has contributed to the development of a commercial bait vaccine for the SAG2 strain in France (5, 6). Several types of vector vaccines, including a canarypox-rabies glycoprotein recombinant vaccine, a recombinant adenovirus- vectored vaccine, and a vaccinia-recombinant glycoprotein (V-RG) virus for wild animals such as raccoon dogs and foxes, have been commercialized. The distribution of ORV has contributed to a rapid decrease in animal rabies in many countries (7–10). As ORV has helped prevent the spread of wild animal rabies in European countries and the USA, the Korean Veterinary Authority has distributed a bait vaccine (V-RG) to high-risk rabies regions. The result of ORV has been a drastic reduction in rabies cases in Korea (11). Nevertheless, human vaccinia infection cases have been reported: two women with a chronic skin disease were exposed to V-RG (12). The women developed vaccinia virus infection on skin of their hand. One of the patients was treated with human vaccinia immune globulin intravenous and antiviral agent. Oral rabies vaccines have not been approved for human use. Therefore, new alternative rabies bait vaccines applicable to pet dogs and wild animals are needed to eradicate rabies. A recombinant RABV strain called ERAG3G was constructed, and efficacy testing of the strain has been conducted in mice. The ERAG3G strain has been considered as oral raccoons vaccine candidate for wild raccoon dogs. In this study, the safety and immunogenicity of the ERAG3G strain were evaluated in wild raccoon dogs.

MATERIALS AND METHODS

Cells and viruses

Murine neuroblastoma (NG108-15) cells were maintained in DMEM supplemented with 5% fetal bovine serum at 37℃ in a 5% CO₂ incubator. NG108-15 cells were used to propagate the recombinant ERAG3G strain, which was constructed in 2011 using a reverse genetic system (2). The ERAG3G strain was deposited in the Korean Veterinary Culture Collection (Accession number: KVCC-VR1500046). After propagation, the ERAG3G strain was titered in 96-well microplates using 10-fold dilutions. The microplates were incubated for 3 days, after discarding supernatant, and then fixed the cells in cold acetone (-20℃) for 20 min. After three successive washes with phosphate-buffered saline (PBS; pH 7.2), the microplates were reacted with a specific monoclonal antibody against RABV for 45 min at 37℃ and then stained with fluorescence isothiocyanate conjugated goat-anti mouse IgG + IgM. After rinsing with PBS, the microplates were air-dried and examined at 400× under a fluorescence microscope (Nikon, Tokyo, Japan). The viral titer determined by a fluorescent antibody test was calculated according to the Reed and Muench method (13). The RABV challenge virus standard (CVS)-11 was widely used in determination of virus neutralizing antibody (VNA). The CVS-11 strain purchased from American Type Culture Collection (ATCC, VR-959) was propagated in baby hamster kidney (BHK)-21 cells and was also titrated in the method mentioned above.

Safety and immunogenicity of the ERAG3G strain in raccoon dogs

This experimental design was submitted to the laboratory animal ethics committee (QIA, Seoul, Korea) and was approved (QIA2013-668). The wounded raccoon dogs were bred for treatment before releasing to where they were caught. Six-month-old raccoon dogs, which were RABV sero-negative, were divided into three groups. Groups 1 and 2 included four raccoon dogs each that were inoculated with the ERAG3G strain (1 ml, 10⁸⁰⁰ fluorescent assay infectious dose, FAID₅₀/ ml) via the oral route using a syringe without a needle and via the intramuscular route, respectively. Two raccoon dogs in the control group remained untreated except for taking blood. The control group was bred with Group 1. After administering the vaccine, all raccoon dogs were kept in a designated place to watch their behavior. All raccoon dogs were monitored daily for adverse effects, including
anorexia, prostration, anxiety, agitation, aggression, and paralysis. Blood was collected from all raccoon dogs, including the control group, 5 and 10 weeks after inoculation.

Serological assay

The VNA titer was determined by a fluorescent antibody virus neutralization (FAVN) test (14). In brief, a positive WHO reference serum adjusted to 0.5 IU/ml was used as the positive control. Serum samples and the positive and negative controls were distributed in four consecutive wells and serially diluted. Then, RABV (CVS-11 strain) containing about 100 FAID50/50 μl was added to each well. A 50 μl aliquot of BHK-21 cells containing 4 × 10^5 cells/ml was added to each well after 60 min of incubation at 37°C, and the microplates were incubated in a humidiﬁed incubator with 5% CO2 at 37°C for 72 h. The microplates were then ﬁxed in cold acetone for 20 min. After three successive washes with PBS, the microplates were reacted with a specific monoclonal antibody against RABV for 45 min 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 examined at 400× under a ﬂuorescence microscope. The comparison of the measured titer of the tested sera with that of the OIE positive standard serum of a known VN titer allowed determination of the VN titer of the tested sera in IU/ml. The comparison to IU/ml was made by using the mean value of the OIE standard serum (14).

RESULTS

Safety and immunogenicity of the ERAG3G strain in raccoon dogs

All raccoon dogs were sero-negative for RABV VNA.
before vaccine administration, but sero-converted 35 days after oral inoculation (Table 1). None of the vaccinated or unvaccinated raccoon dogs exhibited clinical signs of rabies during the experiment. All raccoon dogs in Group 1 that were administered the ERAG3G strain orally developed RABV VNA titers of 7.9~41.6 IU/ml (geometric mean, 19.2 IU/ml) 35 days after inoculation and had a geometric mean VNA titer of 20.6 IU/ml after 70 days. All raccoon dogs in Group 2 that were inoculated intramuscularly with the ERAG3G strain had RABV VNA titers of 0.5~13.7 IU/ml 70 days after inoculation (Fig. 1). Two raccoon dogs in Group 3 remained RABV sero-negative throughout the test, confirming that no contact transmission had occurred between the vaccinated and control animals.

**DISCUSSION**

Raccoon dogs play a key role in transmitting the disease in Northeast Asia, including Korea, and the ferret badger is the major vector species in Taiwan (14~16). Each country has made efforts to control RABV through the mass vaccination of pets and domestic animals. Government policy has led to a significant decrease in human and animal rabies cases in many countries (17). Nevertheless, the ability to completely eliminate rabies is limited because 80% dog vaccination coverage is required for complete eradication (18). Therefore, a new more effective approach is desired to overcome this limitation.

ORV has been developed to replace parenteral vaccination because of its practical advantages, including no need to restrain dogs or risk handling aggressive dogs. ORV can also be applied to wild animals. Large numbers of bait vaccines have been distributed to control and eliminate RABV in wild animals. Many countries have carried out ORV programs with SAG2, a recombinant adenovirus expressing the rabies protein, or V-RG bait (8~10, 19). These programs have contributed to the elimination of rabies in several countries, including Finland, the Netherlands, Italy, Canada, and the USA (20, 21).

Previously, we found that the ERAG3G strain has a glutamic acid (Glu) coding sequence (GAA) at position 333 of the glycoprotein, demonstrating that the ERAG3G strain is non-pathogenic to mice, cats, and dogs (2, 22). However, other researcher reported that amino acid 333 of the G protein is not entirely responsible for the pathogenicity of RABV. For example, RC-HL, a non-pathogenic RABV fixed strain, has an Arg (R) at this position. The RABV G protein is the major determinant of viral pathogenicity and the major protective antigen. It is reported that attenuated RABV strains express higher levels of G protein in the
infected neurons than highly pathogenic street strains (23). Therefore, we assume that the ERAG3G strain may increase the expression of G protein in the infected cells. We also demonstrated that 4- and 6-week-old mice administered the ERA strain at a titer of $10^{6.5} \text{FAID}_{50}/\text{ml}$ via the intramuscular or intracranial route were pathogenic 6 days after inoculation, whereas mice inoculated with $10^{6.5} \text{FAID}_{50}/\text{ml}$ of the ERAG3G strain did not display any clinical signs after a 15-day observation period (24). Another study reported that the ERAG333 strain containing Glu at position 333 was not pathogenic to mice that were > 7 days old (25). Furthermore, orally immunizing mice with the ERAG3G strain induced complete protection (24).

In our study, the safety and immunogenicity of the ERAG3G strain were evaluated in Korean raccoon dogs administered via the oral or intramuscular route. As carnivores are primarily responsible for transmitting rabies, the safety and immunogenicity of an oral vaccine candidate should be evaluated in the target animals. The SAG2 strain is safe based on the absence of adverse clinical signs, salivation, and the absence of replication of the vaccine strain in the brains of vaccinated dogs (25). Our results demonstrate that none of the raccoon dogs inoculated with $10^{6.5} \text{FAID}_{50}/\text{ml}$ intramuscularly or orally showed any adverse effects associated with disease behavior, and that all raccoon dogs administered the ERAG3G strain orally developed high VNA titers of 13.7-41.6 IU/ml 10 weeks after administration. This suggests that raccoon dogs vaccinated orally with the ERAG3G strain are protected from a challenge by virulent RABV, which provides a basis for using a rabies bait vaccine strain. Similar results were reported for raccoon dogs vaccinated with the SAG2 strain; they developed VNA titers of 6.59-19.9 IU/ml 60 days after vaccination (21). In addition, all raccoon dogs inoculated intramuscularly with the ERAG3G strain developed moderate VNA titers of 0.5-13.7 IU/ml after 10 weeks. Raccoon dogs administrated with the ERAG3G strain via oral route showed higher level of VNA titer, indicating that direct contact to target organ, tonsil, enable the inoculated animals to induce better immune response. However, we did not conduct an efficacy test in the target animals. Therefore, further study and a challenge in raccoon dogs administered a bait vaccine containing the ERAG3G strain are needed.

In conclusion, based on the clinical observations the ERAG3G strain was safe and immunogenic in Korean raccoon dogs. A single oral or intramuscular immunization with the ERAG3G strain induced high VNA titers against RABV in raccoon dogs. Therefore, the ERAG3G strain should be sufficient for use as a live vaccine or oral bait vaccine in wild animals.

REFERENCES

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