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# Immunogenicity of an inactivated rabies vaccine for animals derived from the recombinant ERAGS strain

**Purpose:** The aims of the present study were to evaluate the immunogenicity of an inactivated rabies vaccine based on the ERAGS strain.

**Materials and Methods:** The ERAGS virus propagated in Vero cells was inactivated with 3 mM binary ethylenimine for 8 hours. Three types of inactivated rabies vaccines were prepared to determine the minimum vaccine virus titers. Four further types of inactivated rabies vaccines were prepared by blending inactivated ERAGS with four different adjuvants; each vaccine was injected into mice, guinea pigs, and dogs to identify the optimal adjuvant. The immunogenicity of a Montanide (IMS) gel-adjuvanted vaccine was evaluated in cats, dogs, and cattle. Humoral immune responses were measured via a fluorescent antibody virus neutralization method and a blocking enzyme-linked immunosorbent assay.

**Results:** The minimum virus titer of the inactivated rabies vaccine was over  $10^{7.0}$  50% tissue culture infectious doses (TCID<sub>50</sub> values)/mL. Of the four kinds of adjuvants, the IMS gel-adjuvanted vaccine induced the highest mean viral neutralizing antibody (VNA) titers of 6.24 and 2.36 IU/mL in guinea pigs and dogs, respectively, and was thus selected as the vaccine for the target animals. Cats, dogs, and cattle inoculated with the IMS gel-adjuvanted vaccine developed protective VNA titers ranging from 3.5 to 1.2 IU/mL at 4 weeks post-inoculation (WPI).

**Conclusion:** Our data indicate that cats, dogs, and cattle inoculated with an inactivated rabies vaccine derived from the ERAGS strain developed protective immune responses that were maintained to 12 WPI.

**Keywords:** Inactivated rabies vaccine, Dog, Cat, Cattle

## Introduction

The World Health Organization, the Food and Agriculture Organization of the United Nations, and the World Organization for Animal Health (OIE) have adopted a global plan of “zero human rabies deaths by 2030”. To realize this goal, the OIE and its sub-regional offices have run workshops and fora on international standards for the eradication of rabies. Many countries, including South Korea, have been striving to meet these standards. The key elements include improved access to post-exposure prophylaxis, mass dog vaccination, active reporting and surveillance, and education [1]. Of these, dog vaccination is the most cost-effective way to achieve the desired goal [2].

Several types of rabies vaccines have been developed and commercialized in South Korea. The first-generation inactivated rabies vaccine (1945 to 1959) was prepared

from rabbit or calf brains and spinal cord tissue infected with the rabies virus (RABV). The inactivated vaccine imparted only short-term immunity and was associated with adverse effects caused by tissue remnants in the vaccine [3]. The second-generation vaccine (1960 to 1980) was prepared from chicken embryos infected with the Flury low-egg-passage strain. Production of this live rabies vaccine ceased at the time of introduction of the Evelyn-Rokitnicki-Abelseth (ERA) strain (from Canada) in 1974 [4]. The third-generation rabies vaccine was a live attenuated RABV comprising the ERA strain grown in primary porcine kidney cells. This live vaccine has been given to several different animals in South Korea since the 1980s. Recently, the OIE has recommended that cell culture-grown, inactivated rabies vaccines with adjuvants, or RABV glycoprotein vector vaccines, should be given to all dogs and cats, except pregnant animals or those <3 months of age, in all rabies-endemic regions [5]. In line with the OIE recommendation, the use of attenuated rabies vaccine strains (that are potentially virulent) may be further restricted in many countries. On the contrary, inactivated rabies vaccines will become more popular in the veterinary field. Inactivated vaccines are inherently very safe. In South Korea, commencing in the 1990s, several types of inactivated rabies vaccines have been imported and new inactivated vaccines manufactured by Korean companies have been licensed. Most RABV strains used to create inactivated rabies vaccines exhibit neurotropic and virulent characteristics [6]. These properties pose risks to production workers prior to viral inactivation. Therefore, a safe and highly immunogenic rabies vaccine strain is required.

The efficacies of inactivated rabies vaccines are lower than those of live vaccines in terms of inducing protective immune responses. To overcome this issue, selection of a powerful adjuvant has become of prime importance [7]. Aluminum hydroxide gel, the most common adjuvant, has been principally employed [8,9]. Recently, various adjuvants for use in animal vaccines have been developed to promote the maturation of bone marrow-derived dendritic cells, thus inducing strong immune responses [10]. An inactivated rabies vaccine including the Montanide pet gel induced more protection than did a vaccine with aluminum hydroxide gel in dogs and cats [11]. New adjuvants are required to increase the immunogenicity of inactivated rabies vaccines. In our previous study, we generated a recombinant RABV that we termed ERAGS, which was demonstrated to be safe and immunogenic in dogs and cattle [12]. However, the immunogenicity of an in-

activated rabies vaccine based on the ERAGS strain has not yet been investigated.

In this study, we prepared inactivated rabies vaccines based on the ERAGS strain. After selecting an optimal adjuvant, we explored immunogenicity of the vaccine in target animals.

## Materials and Methods

### Viruses and cells

The ERAGS strain generated via reverse genetics served as the seed virus for developing inactivated rabies vaccines [12]. The viral titers of ERAGS propagated in Vero cells (ATCC CCL 81) were measured using an indirect fluorescent assay and expressed as 50% tissue culture infectious doses (TCID<sub>50</sub>) values. Vero cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) with 5% (volume per volume) heat-inactivated fetal bovine serum, penicillin (100 µg/mL), streptomycin (100 unit/mL), and amphotericin B (0.25 µg/mL).

### Inactivation of the ERAGS strain

To propagate the ERAGS strain, Vero cells grown in 850 cm<sup>2</sup> roller bottles were washed 3 times with phosphate-buffered saline (PBS; pH 7.2) and inoculated with 1 mL amounts of the ERAGS strain suspension (10<sup>7.0</sup> TCID<sub>50</sub>/mL). After removing the non-invading viruses, fresh DMEM was added and the bottles incubated for 4 days. The viruses were harvested after three successive freeze-thaw cycles and clarified via centrifugation at 3,000×g for 30 minutes. Bulk viruses were inactivated with 3 mM binary ethylenimine (BEI), and the pH level was adjusted to 7.0 with 1 N NaOH. Inactivation proceeded at 37°C for 8 hours. The remaining BEI was neutralized with 2 mM sodium thiosulfate. To prove that inactivation had occurred, 1 mL of the inactivated RABV suspension was dialyzed against PBS for 24 hours and inoculated into fresh BHK-21 and Vero cells. The two cell types were fixed in cold acetone at 4 days post-inoculation (PI), stained with rabies monoclonal antibodies and a fluorescein isothiocyanate conjugate, and observed under a fluorescent microscope. After confirming viral inactivation, the bulk viruses were used to prepare inactivated vaccines.

### Preparation of experimental vaccines

We prepared three types of vaccines with viral titers of 10<sup>6.0</sup>, 10<sup>7.0</sup>, and 10<sup>8.0</sup> TCID<sub>50</sub>/mL that were inactivated with 3 mM BEI. All were mixed with the Montanide GEL 01 (IMS gel; SEPPIC,

Paris, France) adjuvant prior to determination of the minimum viral titers. Four types of inactivated rabies vaccine were prepared using the following adjuvants according to the manufacturer's indications: Montanide IMS1313 VG N (IMS1313; SEPPIC), IMS gel, Montanide ISA 201 VG (ISA201; SEPPIC), and Montanide ISA 206 VG (ISA206; SEPPIC). The first experimental vaccine consisting of the RABV antigen and IMS1313 adjuvant was blended at a 7:3 ratio via agitation at room temperature. The second vaccine comprised the RABV antigen and IMS gel at a 9:1 weight ratio. The third and fourth vaccines (with the ISA201 and ISA206 adjuvants, respectively) were blended at 1:1 weight ratio at 31°C, yielding water-in-oil-in-water (W/O/W) formulations. After blending, each vaccine was dispensed into sterilized glass bottles and stored at 4°C until use.

#### Fluorescent antibody virus neutralization

The fluorescent antibody virus neutralization (FAVN) test followed the standard OIE procedure [13]. The details have been described previously [12]. The viral neutralizing antibody (VNA) titers of serum samples were expressed in IU/mL by comparing the results to those of the positive standard.

#### Blocking enzyme-linked immunosorbent assay

Serum RABV antibody levels were measured using blocking enzyme-linked immunosorbent assay (B-ELISA) as reported previously [14]. The absorbance value was taken to be inversely proportional to the amount of bound RABV antibodies. In brief, sera were diluted to 1/10th of the original concentration and added to B-ELISA plates coated with the RABV antigen. After incubation for 1 hour at room temperature, the plates were washed 5 times, and 100 µL of anti-RABV conjugate was then added to each well. The plates were incubated at room temperature in the dark for 30 minutes and, after discarding the conjugate, washed 5 times. Finally, 100 µL of substrate solution was added to each well, and color was allowed to develop over 15 minutes. After the addition of 100 µL of stop solution to each well, the absorbance values were read with the aid of an ELISA reader (Tecan, Mannedorf, Switzerland) at 450 nm.

#### Minimum vaccine virus titers

The Animal Ethics Committee of the Animal and Plant Quarantine Agency (Gimcheon, Korea) approved the three experimental designs of this study (approval no., 2016-453, 2017-574, and 2018-816). Three types of inactivated rabies vaccines

were inoculated into guinea pigs to determine the minimum inactivated virus titer required. Guinea pigs were divided into three groups, each containing five animals. Each group was inoculated with 0.5 mL of vaccine intramuscularly (IM). Blood was collected at 4 weeks PI (WPI). All sera were subjected to FAVN and B-ELISA testing.

#### Selection of an adjuvant for the rabies vaccine

Four types of inactivated vaccines were inoculated into mice, guinea pigs, and dogs to identify an optimal adjuvant. Mice, guinea pigs, and dogs were divided into four groups of four animals each. The groups received IMS1313-, IMS gel-, ISA201-, and ISA206-adjuvanted vaccines. Each mouse, guinea pig, and dog were inoculated with 0.2, 0.5, and 1 mL, respectively, of one of the vaccines. Mice and guinea pigs were inoculated IM, whereas dogs were inoculated subcutaneously (SC). Blood was collected from each animal, including control animals, at 4 WPI. Anti-RABV antibody levels in all sera were measured using the FAVN and B-ELISA tests.

#### Vaccine immunogenicity in target animals

Four 1-year-old cats, four 3-month-old dogs, and four 1-year-old cattle seronegative for RABV were inoculated with 1, 1, and 2.5 mL, respectively, of IMS gel-adjuvanted vaccine SC or IM. Two each of cats, dogs, and cattle served as the controls. At 0, 4, 8, and 12 WPI, blood was collected, and the anti-RABV antibodies were measured using FAVN tests and B-ELISA. Animal health was monitored daily for 12 weeks.

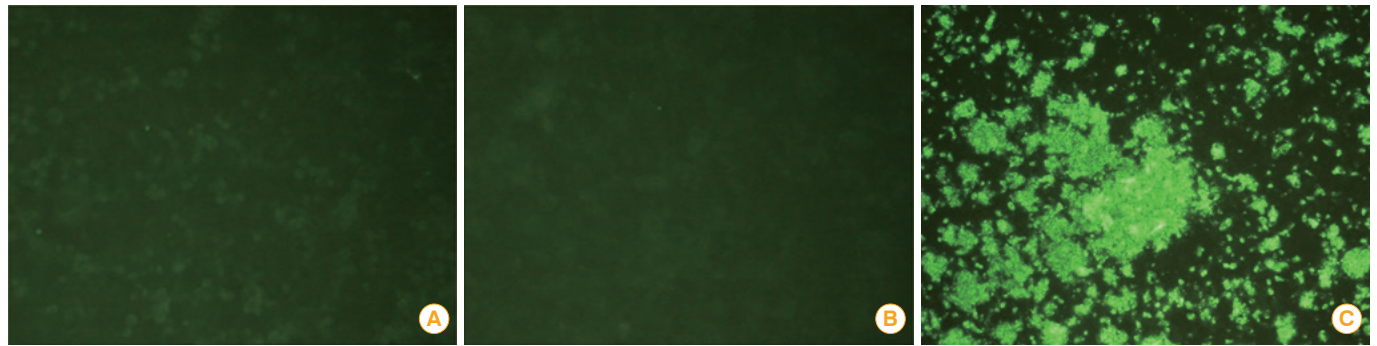
#### Statistical analysis

All data were analyzed using one-way analysis of variance followed by the Tukey post-hoc test. A p-value <0.05 was considered to indicate statistical significance. All statistical analyses were performed with the aid of GraphPad Prism software ver. 5.03 (GraphPad, San Diego, CA, USA).

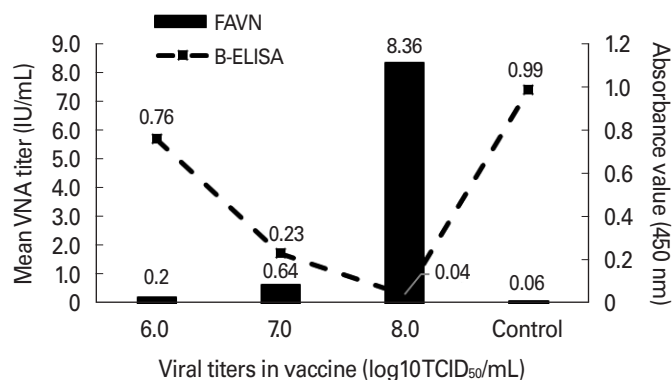
## Results

#### Vaccine preparation

The ERAGS virus was inoculated into Vero cells and harvested at 96 hours PI. After freezing and thawing 3 times, viruses ( $10^{8.0}$  TCID<sub>50</sub>/mL) were prepared and inactivated with 3 mM BEI for 8 hours. After inoculating the inactivated virus into Vero and BHK-21 cells, staining with a specific anti-rabies antibody at 4 days PI revealed no cytoplasmic fluorescence (Fig. 1).



**Fig. 1.** The ERAGS virus was inactivated with 3 mM binary ethylenimine and neutralized with 2 mM sodium thiosulfate. Confirmation of ERAGS virus inactivation (no growth) in Vero cells (A) and BHK-21 cells (B). Live ERAGS growing in Vero cells was used as the positive control (C).



**Fig. 2.** Rabies virus neutralization assay (VNA) titers were measured by the fluorescent antibody virus neutralization (FAVN) test and immune responses were also checked by absorbances from blocking enzyme-linked immunosorbent assay (B-ELISA) of guinea pig sera according to the viral titers of the inactivated rabies vaccines used. TCID<sub>50</sub>, 50% tissue culture infectious doses.

### Determination of minimum virus titers

Inactivated rabies vaccine at three virus titers ( $10^{6.0}$ ,  $10^{7.0}$ , and  $10^{8.0}$  TCID<sub>50</sub>/mL) were mixed with the IMS gel adjuvant prior to the determination of minimum viral titers. As shown in Fig. 2, guinea pigs inoculated with the vaccine at  $10^{8.0}$  TCID<sub>50</sub>/mL developed the highest VNA titer of 8.36 IU/mL and exhibited the lowest serum absorbance of all three vaccine virus titers (FAVN,  $p=0.0006$ ; B-ELISA,  $p<0.0001$ ). No control animal mounted an immune response. Guinea pigs inoculated with the vaccine at  $10^{6.0}$  TCID<sub>50</sub>/mL developed a mean VNA titer of 0.2 IU/mL and exhibited a serum absorbance of 0.76. Guinea pigs inoculated with the vaccine at  $10^{7.0}$  TCID<sub>50</sub>/mL developed a mean VNA titer of 0.64 IU/mL and exhibited a mean serum absorbance of 0.23. Therefore, the minimum vaccine virus titer was determined to be in excess of  $10^{7.0}$  TCID<sub>50</sub>/mL, because a VNA titer of 0.5 IU/mL is characteristic

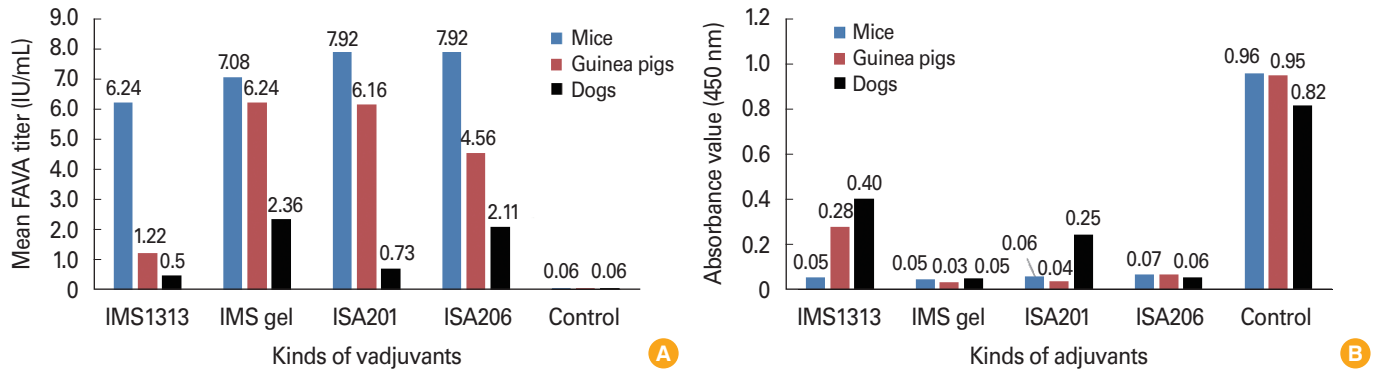
of a protective anti-rabies antibody response.

### Selection of an adjuvant

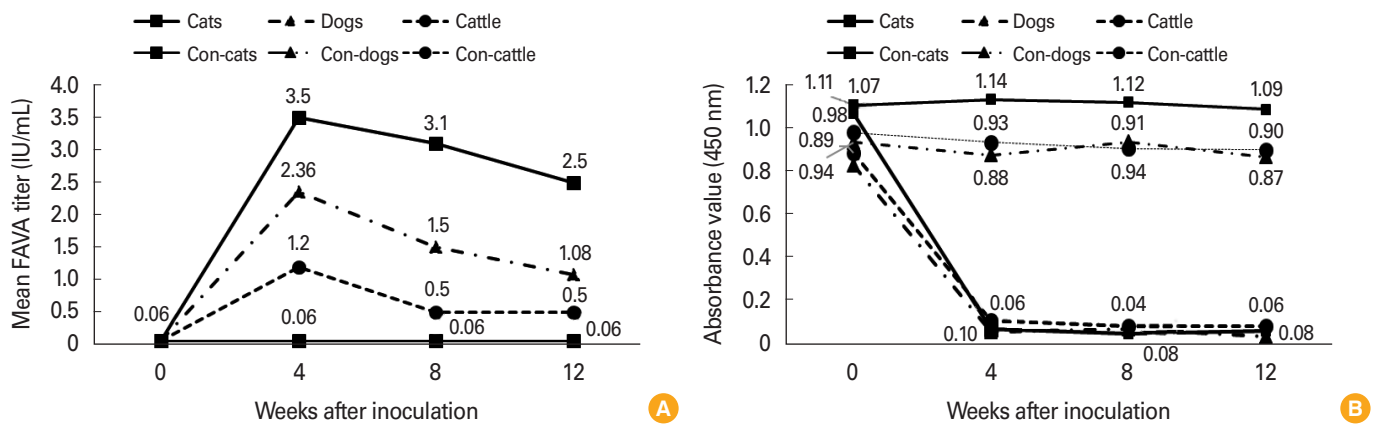
Four inactivated rabies vaccines with a viral titer of  $10^{8.0}$  TCID<sub>50</sub>/mL were prepared using the IMS1313, IMS gel, ISA201, and ISA206 adjuvants; we sought to identify the optimal adjuvant. As shown in Fig. 3A, mice inoculated with the four types of vaccines developed high VNA titers ranging from 6.24 to 7.92 IU/mL. Guinea pigs inoculated with the IMS gel- and ISA201-adjuvanted vaccines developed high VNA titers of 6.24 and 6.16 IU/mL, respectively ( $p=0.0899$ ). Dogs inoculated with the IMS gel-adjuvanted vaccine developed the highest VNA titer of 2.36 IU/mL of all four vaccines ( $p=0.1068$ ). As shown in Fig. 3B, the highest absorbance was observed for guinea pigs inoculated with the IMS1313-adjuvanted vaccine ( $p=0.0026$ ). The rabies vaccines containing the IMS gel and ISA206 adjuvants led to low absorbances of 0.07 or less in mice, guinea pigs, and dogs. The FAVN and B-ELISA tests showed that no control animal developed an immune response. Based on the VNA titer and absorbance results, the IMS gel-adjuvanted vaccine was selected as the inactivated rabies vaccine for target animals.

### Immune responses in target animals

Cats, dogs, and cattle were inoculated with the IMS gel-adjuvanted vaccine to evaluate its immune response. As shown in Fig. 4A, cats, dogs, and cattle developed high mean VNA titers ranging from 3.5 to 1.2 IU/mL at 4 WPI. Although moderate decreases in the mean VNA titers were then evident in all animals, all maintained a protective VNA titer of 0.5 IU/mL up to 12 WPI. All animals produced serum with very low absorbance from 4 to 12 WPI, compared to the control ( $p<0.0001$ ) (Fig. 4B). Non-vaccinated cats, dogs, and cattle remained



**Fig. 3.** The immunogenicity of four types of inactivated rabies vaccines in mice, guinea pigs, and dogs. The mean rabies virus (RABV) antibody titers in vaccinated animals were measured using fluorescent antibody virus neutralization (FAVN) tests (A) and immune responses were checked by blocking enzyme-linked immunosorbent assay (B-ELISA) (B). Rabies vaccines containing the ISA201 or ISA206 adjuvants induced the same mean viral neutralizing antibody (VNA) titers of 7.92 IU/mL in mice; rabies vaccines containing the IMS gel and ISA201 adjuvants induced mean VNA titers of 6.24 and 6.16 IU/mL, respectively, in guinea pigs. The rabies vaccine containing the IMS gel adjuvant induced the highest mean VNA titer of 2.36 IU/mL in dogs. Two rabies vaccines (containing the IMS gel and ISA206 adjuvants) induced low absorbances of 0.07 in mouse, guinea pig, and dog sera.



**Fig. 4.** Immunogenicity of an inactivated rabies vaccine containing the IMS gel adjuvant in dogs, cats, and cattle. The mean rabies virus (RABV) antibody titers were measured using fluorescent antibody virus neutralization (FAVN) tests (A) and mean absorbances were measured by blocking enzyme-linked immunosorbent assay (B).

RABV-seronegative according to the VNA titer and B-ELISA tests over 12 WPI. Although the mean VNA titers measured via FAVN could not be compared to those from B-ELISA, the trends in antibody levels were similar between the two tests at 0, 4, 8, and 12 WPI. In addition, no vaccine-related adverse events were observed over the 12 weeks.

### Discussion

Animal vaccination is key for eradicating rabies worldwide. Vaccination is more cost-effective and simpler than post-exposure prophylaxis in many countries [2]. In South Korea, rabies control programs including mass vaccination have resulted in the absence of rabies cases since 2014 [15]. Live at-

tenuated rabies vaccines based on the ERA strain have contributed to this positive outcome. However, the OIE recommends that live attenuated vaccines should be replaced by inactivated vaccines. Therefore, a novel inactivated rabies vaccine that meets the requirements of vaccine producers and animal owners needs to be developed.

Traditional rabies vaccine strains including CVS-11, Pasteur, Pitman-Moore, and RC-HL strains are inactivated [6]; however, attempted propagation in several cell lines such as BHK-21 and Vero cells failed to produce any cytopathic effect. Moreover, these pathogenic strains are dangerous prior to inactivation. In our previous study, we reported on a genetically modified RABV that we termed ERAGS. This was deemed safe in 4-week-old mice, and multiplex reverse transcription-

polymerase chain reaction can be used to differentiate ERA from ERAGS [16]. Therefore, the ERAGS strain is appropriate for the creation of an inactivated rabies vaccine. In this study, we propagated ERAGS in Vero cells and used BEI to inactivate the RABV antigen because formalin, the most common inactivating agent, is less immunogenic compared to BEI. BEI has been used to inactivate RABV during veterinary rabies vaccine production. We found that 3 mM BEI inactivated RABV within 8 hours at 37°C, thus shorter than the typical 18 hours at 37°C [17].

The virus titer of an inactivated rabies vaccine is closely related to its vaccine efficacy [18]. In this study, we prepared three different antigen concentrations ( $10^{6.0}$ ,  $10^{7.0}$ ,  $10^{8.0}$  TCID<sub>50</sub>/mL before inactivation) and blended them with the IMS gel adjuvant. We found that the inactivated rabies vaccine with an antigen concentration of  $10^{8.0}$  TCID<sub>50</sub>/mL induced the highest VNA titer (8.36 IU/mL) in guinea pigs. However, guinea pigs inoculated with the vaccine with an antigen concentration of  $10^{6.0}$  TCID<sub>50</sub>/mL did not develop the minimally protective VNA titer of 0.5 IU/mL. Thus, the minimum vaccine viral titer should be  $10^{7.0}$  TCID<sub>50</sub>/mL.

If an inactivated rabies vaccine is to be highly immunogenic, it is essential to select an optimal adjuvant. The IMS1313 and IMS gel polymer adjuvants have been used to enhance the safety and efficacy of aqueous vaccines against *Eimeria acervulina* and Japanese encephalitis virus [19,20]. The ISA201 and ISA206 adjuvants, which are continuous aqueous phase W/O/W emulsions, have also been used as adjuvants in foot-and-mouth disease vaccines for cattle [21,22]. We compared the immunogenicity of inactivated rabies vaccines containing four adjuvants developed by SEP-PIC (France) in mice, guinea pigs, and dogs. The RABV VNA titers did not differ significantly among mice immunized with each vaccine type. Although statistical significance was lacking of guinea pigs vaccinated with IMS gel-, ISA201-, and ISA206-adjuvanted vaccines, the former two vaccines induced the highest mean VNA titers of 6.24 and 6.16 IU/mL. The IMS gel-adjuvanted vaccine also induced the highest mean VNA titer (2.36 IU/mL) in dogs. Based on these titers and the B-ELISA absorbances of the sera of the vaccinated animals, we selected the IMS gel as the preferred adjuvant.

We evaluated the immunogenicity of the inactivated rabies vaccine in cats, dogs, and cattle, which are the three species most affected by rabies since 1993 in South Korea [15]. The FAVN and B-ELISA tests showed that all animals developed protective immune responses that persisted to 12 WPI. The

VNA titers tended to reflect animal size, with cats developing higher VNA titers than did dogs or cattle. Several factors such as animal size, age, breed, and sampling time have been reported to affect the immunogenicity of rabies vaccines [23]. Therefore, a single dose for cattle should perhaps be 4 to 5 mL.

In conclusion, we used a safe and well-proliferating virus, the ERAGS strain, to prepare an inactivated rabies vaccine. The VNA titers of guinea pigs inoculated with the inactivated vaccine depended on the vaccine virus titer. Based on the immune responses in mice, guinea pigs, and dogs inoculated with four types of inactivated vaccines, an IMS gel-adjuvanted vaccine was selected for further analysis in target animals. The inactivated rabies vaccine induced protective immune responses in cats, dogs, and cattle. Therefore, the vaccine will contribute to maintaining a rabies-free status in South Korea.

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