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Sindbis virus replicon-based DNA vaccine encoding Rabies virus glycoprotein elicits specific humoral and cellular immune response in dogs

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Summary. – A Sindbis virus (SINV) replicon-based DNA vaccine encoding Rabies virus (RABV) glycoprotein G developed previously (Saxena *et al*., *Vaccine* **26**, 6592, 2008) was used for immunization of dogs against rabies. The intradermal injection of DNA vaccine into external ear generated protective level of virus neutralizing antibodies. The cellular immune response was specific to RABV, in particular by an increase in $CD3^+CD4^+$ and $CD3^+CD8^+$ lymphocytes. This study has demonstrated that the SINV replicon-based DNA vaccine encoding RABV G is capable of inducing the protective level of specific immune response in dogs.

Keywords: Rabies virus; glycoprotein; DNA vaccine; dogs; immune response

Introduction

Rabies, a fatal neurotropic disease affecting all mammals including humans, poses a serious threat in developing as well as in developed countries. In the developing countries, dogs represent a major RABV reservoir causing about 60,000 annual deaths (Tordo *et al*., 2006). Nearly half of these fatalities, e.g. 20,000 to 30,000 people die of rabies only in India. In the developing countries more than 96% of rabies cases in humans are caused by dogs. However, only 30–40% vaccination coverage is established in animals. The main limitation is a high cost of the RABV vaccine prepared from cell culture intended for immunization of poverty-stricken human population and animals as well (WHO, 2006). Therefore, it is desirable to develop a safer, cheaper, and efficacious vaccine for mass vaccination against rabies in dogs.

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Abbreviations: CMI = cell-mediated immune; HCMV = Human cytomegalovirus; LPS = lipopolysaccharide; PBMCs = peripheral blood mononuclear cells; PHA = phytohemagglutinin; p.i. = post immunization; RABV = Rabies virus; VN = virus neutralizing, virus neutralization; RFFIT = rapid fluorescent focus inhibition test; SINV = Sindbis virus

The causative agent of the disease, RABV, belongs to the genus *Lyssavirus,* the family *Rhabdoviridae.* It contains a single-stranded negative-sense RNA genome of about 12 kb in length. RABV protein G is the only glycosylated protein out of 5 proteins expressed by RABV genome. Glycoprotein G is exposed on the surface of viral particle and mediates the virus entry into host cell. Therefore, much attention has been focused on RABV G in the development of a subunit vaccine including DNA vaccine. Previous reports on DNA vaccine against rabies in mice and in other animal species indicated that the mass prophylactic vaccination of the dogs may be performed by DNA vaccine (Xiang *et al*., 1994, 1995; Lodmell *et al*., 1998; Perrin *et al*., 1999; Osorio *et al*., 1999; Gupta *et al*., 2005, 2006; Patial *et al*., 2007). However, clinical trials using DNA vaccinations against various diseases are facing several problems including multiple immunizations with high DNA doses, which are required to achieve protective response especially in big animals (Calarota *et al*., 1998; MacGregor *et al*., 1998; Babiuk *et al*., 2003). Additional problems include potential risk related to the integration of plasmid DNA into host chromosome, tolerance to a DNA vector, and generation of autoimmune diseases (Mor *et al*., 1996; MacGregor *et al*., 1998; Martin *et al*., 1999; Beger *et al*., 2002).

To overcome the difficulties associated with DNA vaccination, an RNA replicon-based vector system based on

SINV (the family *Togaviridae*, the genus *Alphavirus*), has shown considerable promise as a new, effective DNA vaccine vector with increased immunogenicity and biosafety. This system is essentially self-amplifying and self-limiting. Its replicon component consists of plasmid DNA, where SINV replicon sequence is placed under the control of a strong RNA polymerase II promoter derived from the Human cytomegalovirus (HCMV) and the heterologous gene is under the control of the SINV subgenomic promoter (Xiong *et al*., 1989). Replicon systems have been developed for SINV (Xiong *et al*., 1989; Herweijer *et al*., 1995; Hariharan *et al*., 1998; Miller *et al*., 2008; Saxena *et al*., 2008), Semliki Forest virus (Berglund *et al*., 1998), Venezuelan equine encephalitis virus (Lee *et al*., 2003), and Kunjin virus (Anraku *et al*., 2002; 2008). These studies demonstrated that RNA replicon-based DNA vaccines provided higher levels of protective immunity due to the induction of dsRNA-mediated anti-viral pathways (Frolov and Schlesinger, 1994) and significant dose-sparing advantages compared with conventional DNA vaccines (Leitner *et al*., 2003).

In our previous study, we have constructed the SINV replicon-based DNA vaccine encoding RABV G (Saxena *et al*., 2008). Here, we report the immunization of dogs with this DNA vaccine that resulted in an induction of humoral as well as cellular immune response against rabies.

Materials and Methods

DNA vaccine. The SINV-based rabies DNA vaccine contained 5'-UTR, replicase gene, subgenomic promoter and 3'-UTR from SINV under the control of HCMV immediate early promoter. The RABV G was cloned immediately downstream of the SINV subgenomic promoter and upstream of the 3'-UTR. The vector backbone contained pUC ori site and kanamycin resistance gene for replication and selection in E. coli, respectively (Saxena *et al*., 2008). The DNA vaccine was isolated using EndoFree plasmid Giga kit (Qiagen) following manufacturer's instructions. The concentration of the plasmid DNA was estimated by the measurement of A_{260}. The DNA used for immunization was ethanol precipitated, resuspended in saline at the concentration of 1 mg/ml, and stored at -20°C until used.

Immunization of dogs. Before vaccination, 4–6 week-old clinically healthy puppies were blood-sampled and their sera were tested for presence of RABV antibodies in virus neutralization (VN) test using the rapid fluorescent focus inhibition test (RFFIT). Two seronegative dogs (R1312 and R1313) were injected intradermally with 50 µg of DNA vaccine in saline in each ear pinna. The control dog was injected with an empty vector. The booster dose was given on day 21 post immunization (p.i.). The blood samples were taken on day 21, 28, and 35 p.i. and sera were tested for the presence of VN antibodies using RFFIT. The experimental animal manipulation was done according to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Environment and Forests, Government of India.

Titration of VN antibodies. VN antibody titers in sera from immunized and control dogs were determined using RFFIT as previously described (Patial *et al*., 2007) using a WHO standard as reference. Antisera with known levels of International Units (IU)/ml of VN antibodies against RABV were included as the positive control in all assays. In our assays, a titer equivalent to or above 0.5 IU/ml indicated a successful vaccination or immunologic response according to the WHO recommendations (WHO, 2006).

Assay of cell-mediated immune (CMI) response. Two weeks after booster injection, peripheral blood mononuclear cells (PBMCs) were isolated from the immunized and control dogs and CMI responses were analyzed by RABV antigen-specific lymphocytes proliferation assay and phenotyping of effector cells after *in vitro* stimulation with inactivated purified RABV antigen.

The proliferation assay was performed using MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) dye following the method described elsewhere (Mosmann, 1983; Bounos *et al*., 1992). The PBMCs were stimulated with the inactivated purified RABV antigen (10 µg/ml) or lipopolysaccharide (LPS) or phytohemagglutinin (PHA) (10 µg/ml) (positive control) or medium alone (negative control) at 37ºC under 5% CO_2. The stimulation index (SI) was calculated as a ratio of A_{550} with antigen stimulation to background A_{620} without antigen stimulation.

Phenotyping of lymphocytes by flow cytometry. PBMCs were stimulated *in vitro* with the inactivated purified RABV antigen for 48 hrs. The cells were stained with a triple color reagent (Serotech) e.g. cocktail of anti-dog CD3-FITC/CD4-RPE/CD8-Alexa Fluor 647-labeled monoclonal antibodies specific for the cell surface antigens CD3, CD4, and CD8. Non-stimulated cells from each dog were also stained. Cocktail of labeled antibodies was mixed with about 10^6 cells and incubated for 30 mins. Stained cells were washed twice with PBS-BSA and resuspended in PBS containing 1% paraformaldehyde. The numbers of $CD3^+CD4^+$ and $CD3^+CD8^+$ cells from duplicate samples collected from immunized and control dogs were acquired from 10,000 cells/sample using BD FACS Calibur flowcytometer (BD Biosciences). Acquired data were analyzed using BD CellQuest program (BD Biosciences).

Results

RABV-specific humoral response in dogs immunized with the DNA vaccine

To assess an immunogenicity of the DNA vaccine in dogs, sera from the immunized dogs were analyzed for the presence of VN antibodies and compared with the serum of control dog. RABV-specific seroconversion was observed in the immunized dogs assayed in VN test using RFFIT as early as on the day 21 p.i. There was no seroconversion in the control dog (Fig. 1). The VN ability of induced antibodies in the immunized dogs was higher than 0.5 IU/ml and increased 2–4 folds after a booster immunization dose (Fig. 1).

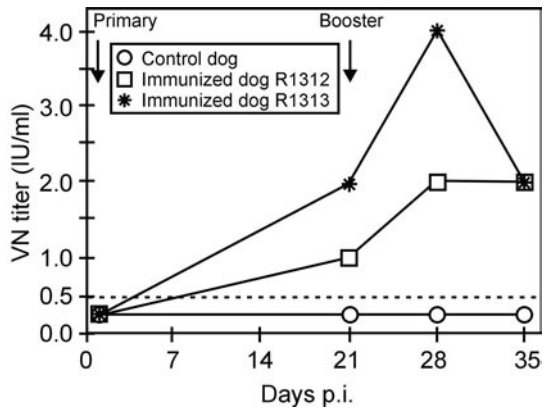

Fig. 1

Serum VN antibody in dogs immunized with the DNA vaccine

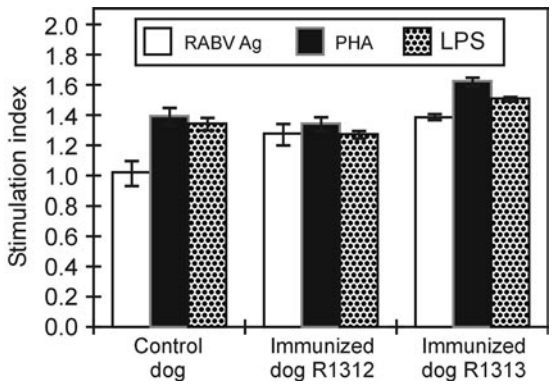

Fig. 2

Proliferative responses of lymphocytes in dogs immunized with the DNA vaccine

CMI response in dogs immunized with the DNA vaccine

RABV-specific lymphocyte proliferative responses of PBMCs were detected in the immunized dogs after *in vitro* stimulation of PBMCs with inactivated purified RABV antigen (Fig. 2). Low or non-significant RABV-specific proliferation was detectable also in the control dog. The proliferative response of immunized and control dogs with non-specific stimulator (PHA and LPS) confirmed that the cells were healthy and competent to proliferate. It was concluded that the proliferative response of lymphocytes primed with the DNA vaccine was RABV-specific.

To characterize the RABV-specific stimulation of lymphocytes in immunized dogs, the stimulated lymphocytes were phenotypically characterized for the components of immune effectors ($CD4^+$ and $CD8^+$) using a set of different monoclonal antibodies against CD3, CD4, and CD8 surface markers on lymphocytes and compared with their respective non-stimulated controls. The numbers of $CD3^+CD4^+$ and $CD3^+CD8^+$ cells were significantly higher in the immunized dogs than in control dog. *In vitro* stimulation with inactivated purified RABV antigen increased the population of $CD3^+CD4^+$ and $CD3^+CD8^+$ effector cells in the immunized dogs, what indicated the priming with RABV antigen. There was no increase in the population of $CD3^+CD4^+$ and $CD3^+CD8^+$ cells in lymphocytes of control dog after *in vitro* stimulation (Fig. 3).

Discussion

The principal aim of this study was to explore and evaluate the immune response in dogs induced by a SINV-based DNA vaccine expressing RABV G. The individual neutralizing antibody titer of both vaccinated dogs exceeded 0.5 IU/ml analyzed on the day 21 p.i. According to the WHO recommendations, the VN titer >0.5 IU/ml indicates protective status against rabies (WHO, 2006). In contrast, the serum of the control dog exhibited VN titer below 0.5 IU/ml. Further, strong VN response was seen in both vaccinated dogs 7 days after the booster injection. We detected 2-fold increase in VN antibody titers that were maintained above the protective threshold (0.5 IU/ml) up to the day 35 p.i. Results of our previous study (Patial *et al*., 2007) and results of others (Perrin *et al*., 1999; Lodmell *et al*., 2003) with DNA vaccination against rabies in dogs corresponded with the present results showing that protective levels of VN antibody were produced after a single shot of DNA vaccine. In this study we have not challenged the dogs with RABV due to the biosafety reasons.

The increase in lymphocyte proliferation and number of $CD4^+$ and $CD8^+$ lymphocytes after *in vitro* stimulation with inactivated purified RABV antigen indicated RABV-specific CMI response in both vaccinated dogs. It has been demonstrated by several studies that SINV-based DNA vaccine vector induced apoptosis in transfected cells (Kohno *et al*., 1998; Leitner *et al*., 2000; Saxena *et al*., 2008). The apoptotic cells are easily picked up by dendritic cells for subsequent processing and class I-restricted presentation to $CD8^+$ cells resulting in an enhanced CMI response (Albert *et al*., 1998). In addition, dsRNA intermediates generated during the alphavirus replicon-mediated expression are a "danger signal" that serves as an adjuvant to the T-cell-specific stimulus of encoded antigen (Leitner *et al*., 1999; Saxena *et al*., 2008). Based on these evidences, we speculate that the enhanced CMI response elicited in the immunized dogs may be due to the induced apoptosis and/or the dsRNA adjuvant effects.

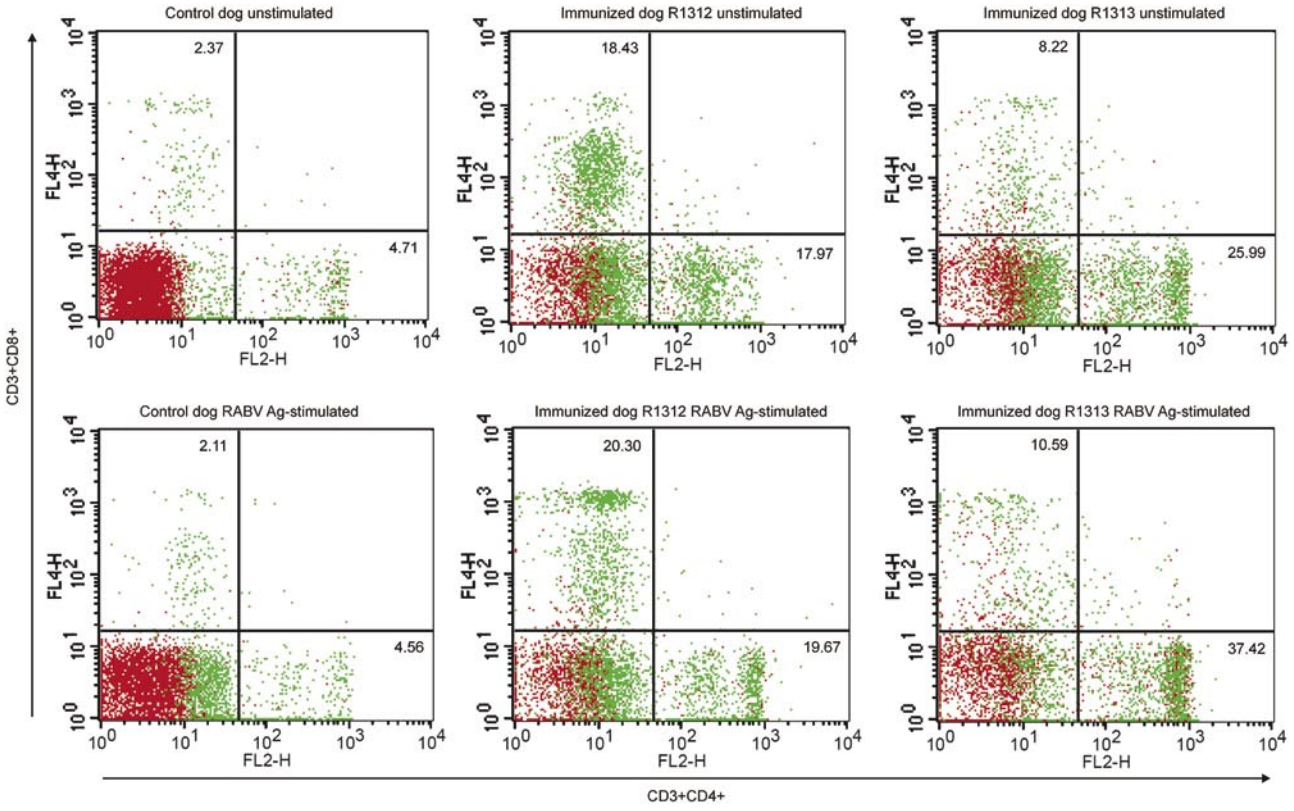

Fig. 3

CMI response in dogs immunized with the DNA vaccine by flow cytometry

This study reports the successful application of a SINV-based rabies DNA vaccine expressing RABV G encoded under the SINV subgenomic promoter for eliciting sufficient levels of VN antibodies and cellular immune response specific to RABV. The immunization of dogs with this vaccine indicated dose-sparing advantages, because the immunization dose was similar to the one used in mice. Furthermore, the non-replicating character of this DNA vaccine has a superior safety profile desirable for the vaccine. Similarly to other DNA vaccines, it also offers significant advantages over the cell culture vaccine including (i) the capacity for rapid production scale-up, (ii) low cost of production compared to the cell culture vaccines, and (iii) no need for a cold transport during distribution. Taken together, this RABV vaccine may be an effective alternative strategy for vaccination of the dogs and can be considered as a promising approach for mass vaccination of puppies and adult dogs in developing countries.

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