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Construction and Immunogenicity of a Recombinant Pseudorabies Virus Expressing the Rabies Virus Glycoprotein and EGFP

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Abstract: The rabies virus glycoprotein is an efficient vaccine antigen particularly for the oral vaccine of wildlife. Pseudorabies virus contains many non-essential genes and was usually used to construct recombinant vaccines as a viral vector to deliver foreign genes into the target animals. In this study, a recombinant pseudorabies virus rPRV/SRV9G/EGFP, expressing EGFP and rabies virus (SRV9 strain) glycoprotein was constructed by homologous recombination in the *UL21* gene. The immunogenicity was evaluated as a vaccine in mice. In this trial, thirty mice were administered with the recombinant vaccine orally. This recombinant virus was demonstrated to be safe for mice by oral administration and could induce neutralizing antibody level above 0.5 IU mL^{-1} at 3 weeks after the vaccination. The results suggest that rPRV/SRV9G/EGFP was an effective oral vaccine candidate against rabies and *UL21* gene could be a target insertion area for foreign genes.

Key words: Rabies Virus (RV), Pseudorabies Virus (PRV), *UL21* gene, SRV9 glycoprotein, oral vaccine, homologous recombination

INTRODUCTION

Rabies is a deadly disease that continues to menace all mammals (including humans) in many areas of the world especially Asia and Africa (Hu *et al*., 2006). Humans and animals are usually infected with the rabies virus through a bite or a scratch by a rabid animal. In Africa and Asia, the vast majority of human rabies is associated with virus transmission from dogs although, wild carnivores and bats are also important reservoirs (WHO, 2005). Cell culture vaccines and anti-rabies virus immunoglobulins cost too much while oral vaccine is cheap and convenient to control the dog and wildlife rabies. So, oral vaccination is an important part of wildlife rabies control programs. A number of live vaccines such as some attenuated rabies virus strains (ERA/SAD/SAG) and recombinant vaccinia (V-RG) viruses have been used successfully in oral vaccination field programs to limit the spread of rabies in coyotes, foxes and raccoons in North America and Western Europe (Kieny *et al*., 1990; Brochier *et al*., 1990; Cliquet and Barrat, 2008).

The rabies virus genome produces five monocistronic mRNAs encoding Nucleoprotein (N), Phosphoprotein (P), Matrix protein (M), Glycoprotein (G) and viral RNA-dependent RNA polymerase (L), respectively (Wunner and Larson, 1988). The G protein is a 65 kDa type I transmembrane protein consisting of an extracellular domain, a transmembrane domain and a cytoplasmic domain. It is the only protein on the surface of mature virus and plays an important role in virus infection and also a major antigenic stimulus of the host immune system inducing neutralizing antibodies (Cox and Dietzschold, 1977).

Pseudorabies Virus (PRV), a member of herpesvirus type I has a broad host range including sheep, cattle, dogs and rodents (Klupp *et al*., 2004). PRV contains many non-essential genes such as Thymidine Kinase (TK), Protein Kinase (PK), Glycoprotein E (gE), glycoprotein I (gI) and glycoprotein G (gG) which make insertion of foreign genes feasible (Klupp *et al*., 2004; Olsen *et al*., 2006). The PRV Bartha K-61 strain, used in the trial has

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been used as a live vaccine against Aujeszky's disease in swine for decades and has a good safety record. Because of so many advantages described before, the PRV was usually used to construct recombinant vaccines as a viral vector to deliver foreign genes into the target animals (Liu *et al*., 2008; Yuan *et al*., 2008). The UL21 protein of PRV is a capsid-associated protein involved in capsid maturation during cleavage and encapsidation of the viral genome and deletion of the pUL21 could result in a drastic decrease in the incorporation of the pUL46, pUL49 and pUS3 tegument components into mature virions (De Wind *et al*., 1992; Wagenaar *et al*., 2001; Michael *et al*., 2007). The PRV Bartha K-61 strain has 3 amino acid mutations in UL21 protein that affect the virus virulence and ability to spread through neural circuits (Klupp *et al*., 1995). The virus lacking *UL21* gene could be propagated on non-complementing cells and nasal mucosa explants indicating that UL21 protein are not required for viral replication in cell culture (Wagenaar *et al*., 2001; Klupp *et al*., 2005) and could be replaced by exogenous proteins.

In this study, the researchers generated a recombinant pseudorabies virus replaced the *UL21* gene by a double expression cassette of EGFP and rabies virus SRV9 glycoprotein and its ability to induce protective immunity against virulent challenge was tested on BALB/c Mouse Model.

MATERIALS AND METHODS

Cells and virus: BHK-21 cells were grown in Dulecco's Modified Eagle's Medium (DMEM, Gibco BRL Life Technology Inc) supplemented with 100 IU penicillin mL^{-1}, 100 mg streptomycin mL^{-1} and 3% (v/v) Fetal Bovine Serum (FBS, Gibco) at 37°C in 5% CO_2. PRV vaccine strain Bartha-K61 was maintained in the laboratory, propagated and titrated in BHK-21 cells. The pEGFP-N1 Vector was purchased from CLONTECH Laboratories, Inc. The G gene of RV strain SRV9 was amplified and cloned into pMD18-T vector as described before (Hu *et al*., 2006).

Construction of transfer plasmids: To construct the SRV9 glycoprotein expressing cassette, the *SRV9 G* gene was cut from pMD18-T-SRV9G and inserted into the pcDNA3.1(+) vector and the name of the new plasmid was termed pcDNA-SRV9G. To produce the double expression cassette, the EGFP expressing cassette was cut from pEGFP-N1 vector and replaced the neomycin resistance gene of the pcDNA-SRV9G. The genomic DNA of PRV Bartha-K61 strain was extracted and purified as described previously (Jestin *et al*., 1990). The two fragments of the *UL21* gene (GenBank accession NC_006151) were obtained by Polymerase Chain Reaction (PCR) amplification from PRV Bartha K-61 strain genomic DNA as the homologous recombinant arms with two pairs of specific primers as Table 1. Then, to construction the transfer plasmid, the double expression cassette, flanked by the two fragments of *PRV UL21* gene was cut from the pcDNA3.1-SRV9G-EGFP and inserted into the pPoly2 vector.

Table 1: Primers used to amplify the homologous recombinant arms flanked *UL21* gene

Primers	Primer sequence[a] (5'-3')
F1	TTGAGTACCAGAGCACGAT
R1	ATGCACTCGACGAGGTAG
F2	ATGCGGATGCTCGTGAAC
R2	TATTGAGGACGATGGAGATGT

Generation of the recombinant rPRV/SRV9G/EGFP virus: The recombinant rPRV/SRV9G/EGFP was generated by co-transfection with the genomic DNA of PRV Bartha K-61 and plasmid pUL21-SRV9G-EGFP via homologous recombination using FuGENE® HD transfection reagent, according to manufacturer's instructions. At 3 days post transfection, virus-containing supernatant was collected and subcultured into BHK-21 cells for plaque purification. After 2 h incubation with recombinant virus containing supernatant, cell monolayers were overlaid with 2% low gelling temperature nutrient agar containing 10% (v/v) heat-inactivated Fetal Bovine Serum (FBS) (Invitrogen Corporation, San Diego, CA, USA), 4 mmol L^{-1} L-glutamine, 1.97 g L^{-1} $NaHCO_3$ for plaque isolation. Potential positive recombinants were screened by their green-fluorescing plaque phenotype under a fluorescence microscope. The green-fluorescing cell foci was picked by aspiration and seeded on BHK-21 cells. After six rounds of screening, plaques showing green fluorescence by fluorescent microscopy were harvested. Presence of SRV9 glycoprotein was detected by PCR amplification using specific primers for *SRV9 G* gene. Fg: 5'-CCGGAATTCCC ACCATGGTTCCTCAGGCTCTCC-3',Rg:5'-CGCGGATCC TTACAGTCTGGTCTCACCC-3'.

Western blot analysis of the glycoprotein expressed in BHK-21 cells: Recombinant virus rPRV/SRV9G/EGFP and PRV Bartha-K61 were grown in BHK-21 cells for 72 h. About 20 μL cell lysates were subjected to SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE) followed by immunoblotting onto nitrocellulose membranes as described by Sui *et al*. (2010). Nonspecific antibody-binding sites were blocked with 5% Dried Skim Milk (DSM) in Phosphate-Buffered Saline (PBS). Mouse anti-rabies virus glycoprotein monoclonal antibody and Horseradish Peroxidase (HRP)-conjugated goat anti-mouse IgG (SIGMA, St.Louis, MO, USA) were respectively used as the primary and secondary antibody at dilutions specified by the manufacturer. 3, 3'-Diaminobenzidine tetrahydrochloride (DAB) was the substrate.

Oral vaccination of rPRV/SRV9G/EGFP on mice: Ninety healthy 4 weeks old mice with no history of vaccination against pseudorabies or rabies were purchased from Animal Center of Institute of Changchun Biological Products, Changchun, China. They were randomly divided into three groups of 30 each. All mice were inoculated by oral route. Group 1 and 2 were respectively inoculated once with 1 mL of medium containing 6×10^6 Plaque Forming Units (PFU) of rPRV/SRV9G/EGFP or PRV. Group 3 was served as a negative control and inoculated with 1 mL of medium without any virus following the same administration. After immunization, serum samples were collected from the tail vein at various time-points for serological tests.

Neutralizing antibodies against rabies viruses: Mice sera were inactivated at 56°C for 30 min. Rabies virus neutralizing antibody was determined by the Fluorescent Antibody Virus Neutralization (FAVN) test as described previously (Hu *et al*., 2006).

Data analysis: Data were analyzed by one-way ANOVA using the SPSS 13.0 Software package (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Construction and identification of recombinant pseudorabies virus: After 6 cycles plaque isolation, almost all the cell foci showed green fluorescence under the fluorescence microscope (Fig. 1). In BHK-21 cells, the growth assay showed that there were no significant differences in plaque size and titers between the recombinant and parental virus. It indicated that the insertion of foreign genes in the *UL21* gene does not affect the replication of PRV.

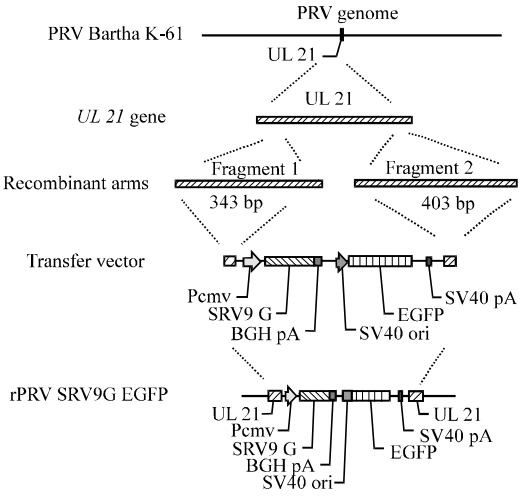

Fig. 1: Schematic diagram of the generation of recombinant PRV

Rabies virus glycoprotein expression of rPRV/SRV9G/EGFP in BHK cells: The *SRV9* glycoprotein gene was homologous recombined into the PRV genome that was verified by PCR with specific primers for *SRV9 G* gene. The Western blot results also demonstrated correct expression of SRV9 glycoprotein (65 kDa) in the identified recombinant virus (Fig. 2).

Neutralizing antibody against rabies: Neutralizing antibody levels against rabies virus of group 1 mice were >0.5 IU mL^{-1} from the 3rd week onward and lasted for >3 months keeping this level. Data represent the mean value with standard deviation (Fig. 3).

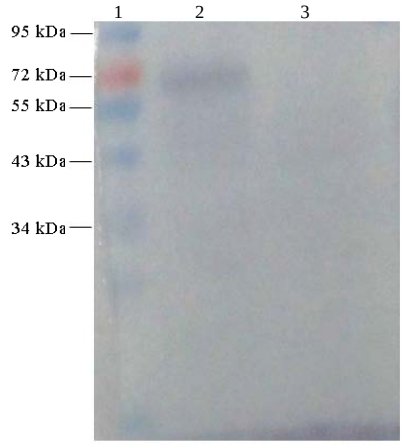

Fig. 2: Western blot analysis of SRV9 glycoprotein expressed by rPRV/SRV9G/EGFP. Lane 1: PageRuler™ Prestained Protein Ladder (NEB Inc, England), Lane 2: BHK cell infected with rPRV/SRV9G/EGFP; Lane 3: BHK cell infected with Bartha K-61

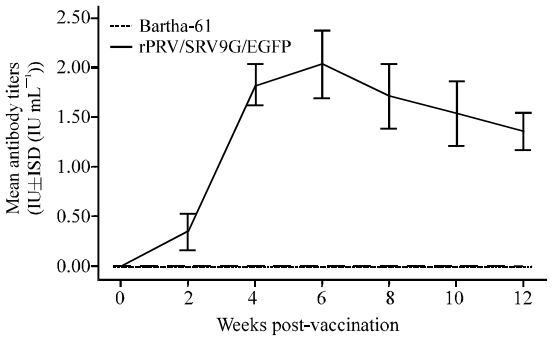

Fig. 3: Neutralizing antibody levels induced by rPRV/SRV9G/EGFP in mice

The development of oral vaccines to control rabies in China and other developing countries is necessary and urgent. In the recent years, there are several types of live vectors used in construction of rabies recombinant vaccines. In the North America and Western Europe, the Vaccinia virus-based rabies vaccine has been used to control wildlife rabies. However, in the immunodeficient people, contact with the Vaccinia virus-based rabies vaccine could cause adverse reactions (Brochier *et al*., 1990). Human adenovirus type 5 used for rabies vaccine development also has safety problems in human (Yarosh *et al*., 1996). Pseudorabies virus has long been explored for vaccine development (Qian and Li, 2004; Hong *et al*., 2007). The PRV strain Bartha K-61 is an approved vaccine strain for prevention of porcine pseudorabies in China. It should be safe for all domestic species as well as pigs. The researchers constructed a recombinant pseudorabies expressing fusion of the ectodomain of the rabies virus glycoprotein with EGFP which could effectively induce the rabies virus neutralizing antibody in dogs (Yuan *et al*., 2008). However, compared to the commercial inactivated rabies vaccine, the neutralizing antibody level is much lower. The relatively low response was probably related to the ectodomain of the glycoprotein and the fusional expression. So, in this study, the resaerchers amplified the whole glycoprotein of SRV9 and constructed a double expression cassette individually expressing rabies virus glycoprotein and EGFP. It induced higher neutralizing antibody level than that of the fusional expression recombinant pseudorabies and provided full protection against rabies virus challenge. The results indicated that the transmembrane region of rabies virus glycoprotein should be necessary in exhibiting antigens of the recombinant virus. Neutralizing antibody was monitored for 3 months at the end of which VNA titers were still at a high level above 0.5 IU mL^{-1}.

UL21 locus of PRV Bartha K-61 contains seven point mutations that induced 3 amino acid mutations of UL21 protein (Klupp *et al*., 1995). These mutations result in inefficient tegument assembly in PRV Bartha K-61 (Michael *et al*., 2007) and such a defect may diminish the infectivity of transmitted virions. In this study, researchers amplified two fragments of *UL21* gene used as a pair of homologous recombination arms. By homologous recombination, we inserted a double expression cassette of SRV9 glycoprotein and EGFP into the PRV Bartha K-61 genomic DNA and obtained a recombinant virus (Fig. 4). For a recombinant virus vaccine, it is important to verify that the insertion of foreign genes in the parental virus genome and the expression of foreign protein could not adversely affect

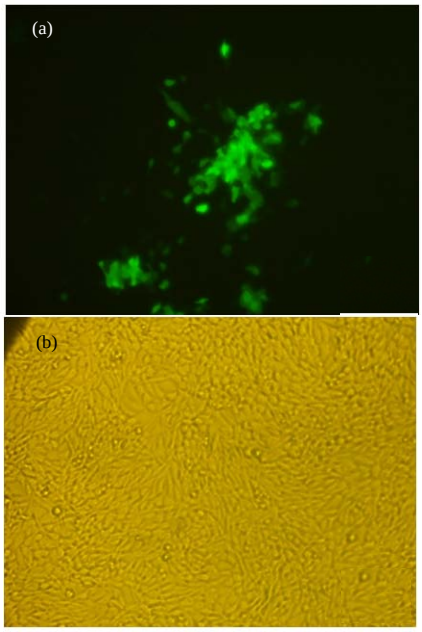

Fig. 4: Expression of EGFP in BHK cell foci by fluorescence microscopy; a) rPRV/SRV9G/EGFP-infected cells 36 h post-infection and b) Normal control cells

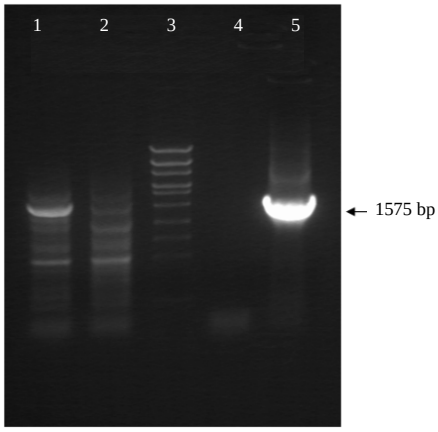

Fig. 5: SRV9 glycoprotein gene in the recombinant rPRV/SRV9G/EGFP was detected by PCR amplification; Lane 1: rPRV/SRV9G/EGFP; Lane 2: PRV; Lane 3: 6280 marker; Lane 4: Negative control; Lane 5: Positive control

viral growth. The growth assay showed that the recombinant viruses replicated in cells culture as efficiently as PRV vaccine strain Bartha K-61 and the final virus yields were very similar. The *UL* gene has not been reported in development of recombinant PRV vaccines. The results showed that it was feasible to manipulate this region for insertion of foreign gene (s) (Fig. 5).

CONCLUSION

The data in this trial shows that rPRV/SRV9G/EGFP provides long term, safe and economical immunization in mice. The research also suggests that Bartha K-61 might be used as vector to develop new types of oral vaccines and *UL21* gene could be a target insertion area for foreign genes.

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