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Two potential recombinant rabies vaccines expressing canine parvovirus virion protein 2 induce immunogenicity to canine parvovirus and rabies virus

Jun Luo [a,b,1], Hehe Shi [a,b,1], Yeping Tan [c], Xuefeng Niu [a,b], Teng Long [a,b], Jing Zhao [a,b], Qin Tian [a,b], Yifei Wang [a,b], Hao Chen [a,b], Xiaofeng Guo [a,b,*]

[a] College of Veterinary Medicine, South China Agricultural University, Guangzhou, China
[b] Key Laboratory of Zoonosis Prevention and Control of Guangdong Province, PR China
[c] Institute of Veterinary Medicine, Jiangsu Academy of Agricultural Sciences, Key Laboratory of Veterinary Biologicals Engineering and Technology, Ministry of Agriculture, National Center for Engineering Research of Veterinary Bio-products, Nanjing 210014, China

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ABSTRACT

Both rabies virus (RABV) and canine parvovirus (CPV) cause lethal diseases in dogs. In this study, both high egg passage Flury (HEP-Flury) strains of RABV and recombinant RABV carrying double RABV glycoprotein (G) gene were used to express the CPV virion protein 2 (VP2) gene, and were designated rHEP-VP2 and, rHEP-dG-VP2 respectively. The two recombinant RABVs maintained optimal virus titration according to their viral growth kinetics assay compared with the parental strain HEP-Flury. Western blotting indicated that G protein and VP2 were expressed *in vitro*. The expression of VP2 in Crandell feline kidney cells post-infection by rHEP-VP2 and rHEP-dG-VP2 was confirmed by indirect immunofluorescence assay with antibody against VP2. Immunogenicity of recombinant rabies viruses was tested in Kunming mice. Both rHEP-VP2 and rHEP-dG-VP2 induced high levels of rabies antibody compared with HEP-Flury. Mice immunized with rHEP-VP2 and rHEP-dG-VP2 both had a high level of antibodies against VP2, which can protect against CPV infection. A challenge experiment indicated that more than 80% mice immunized with recombinant RABVs survived after infection of challenge virus standard 24 (CVS-24). Together, this study showed that recombinant RABVs expressing VP2 induced protective immune responses to RABV and CPV. Therefore, rHEP-VP2 and rHEP-dG-VP2 might be potential combined vaccines for RABV and CPV.

1. Introduction

Rabies virus (RABV) belongs to the *Rhabdoviridae* family, a single-strand, negative-sense RNA virus [1]. RABV causes rabies in many species and leads to 100% lethal encephalitis. Rabies is still an important zoonosis that causes numerous human deaths, most of which occur in developing countries [2]. Rabies infection in humans can occur by a scratch or bite from infected animals. An effective vaccine against RABV has an important role in controlling the spread of rabies [3]. Recently, several methods have been used to explore novel RABV vaccine candidates to improve vaccine immunogenicity with reduced cost. Immune molecules to enhance immunity and attenuate virulence *in vivo* were expressed in several RABV candidate strains and compared with the parent strain [4–7]. Virus-like particles of RABV and other G proteins were demonstrated to be an effective and safe vaccine candidate that could protect the host from RABV infection [8,9]. A previous research has indicated that RABV could be inactivated with hydrogen peroxide, a cheap and safe alternative to beta-propiolactone [10]. Although many rabies vaccines were licensed, the expense of these vaccines limits their use in developing countries.

The genome of RABV encodes five structural proteins from 3′ to 5′ consisting of a nucleoprotein (N), phosphoprotein (P), matrix-protein (M), glycoprotein (G) and RNA-dependent RNA polymerase (L). G is the main antigenic protein that induces virus-neutralizing antibodies (VNA) [11]. A recent study indicated that a pseudotyped baculovirus expressing G induced high levels of antibodies that conferred 100% protection in mice [12]. Rabies virus carrying two copy of G induced higher VNA in mice and dogs compared to that carrying one copy of G [13]. Many studies have reported that RABV

* Corresponding author at: College of Veterinary Medicine, South China Agricultural University, Guangzhou, China.
E-mail address: xfguo@scau.edu.cn (X. Guo).
[1] Both Jun Luo and Hehe Shi contributed equally to the work.

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had been used to express exogenous proteins between G and L as well as N and P [14–16]. Some immune-related genes were inserted between G and L leading to enhanced immunogenicity and viral attenuation in the host [5,7,17,18]. Therefore, RABV can be a useful vector to express foreign genes [19].

Canine parvovirus (CPV) is highly infectious and causes a high rate of morbidity and death in dogs [20]. CPV is a single-strand, negative-sense DNA virus of approximately 5.2 kb [21]. Its genomic DNA encodes two structural virion proteins (VP1 and VP2) and two nonstructural proteins (NS1 and NS2) [22,23]. VP2, the major capsid protein, is the main antigenic protein that induces antibodies to prevent the infection of CPV in dogs. In addition, VP2 is essential for determining virus pathogenicity and virus self-assembly, by forming virus-like particles without genomic DNA [24,25]. Moreover, various types of CPV (CPV-2, CPV-2a, CPV-2b and CPV-2c) caused by amino acid changes in VP2 have been reported [21,26]. Changes of five amino acids in VP2 were observed between CPV-2 and CPV-2a while two substitutions occurred at 426 and 555 amino acids between CPV-2a and CPV-2b [25]. CPV-2a, CPV-2b and CPV-2c are distributed worldwide, but CPV-2a is the main strain epidemic in China [27]. Of note, CPV-2a can infect cats as well as dogs [28].

In this study, vaccine candidate strain high egg passage Flury (HEP-Flury) of RABV was used as a vector to express CPV VP2 (from CPV-2a) to construct a recombinant vaccine. The characteristics of the recombinant RABVs (rRABVs) carrying VP2 were determined *in vitro* and *in vivo*. The data indicate that both rHEP-VP2, HEP-Flury carrying VP2, rHEP-dG-VP2, HEP-Flury carrying double G and VP2, could induce high levels of antibodies against both RABV and CPV. Furthermore, rRABVs induced a higher virus titer *in vitro* assessed by a growth kinetics assay. Therefore, rRABVs carrying VP2 may be used as multivalent vaccines against RABV and CPV.

2. Materials and methods

2.1. Cells, viruses, antibodies and animals

BSR cells, a cloned cell line derived from baby hamster kidney (BHK-21) cells, and Crandell feline kidney (CRFK) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Invitrogen, China) with 10% fetal bovine serum (FBS). HEP-Flury was propagated in BSR cells. rRABVs (rHEP-VP2 and rHEP-dG-VP2) were rescued in BHK-21 cells in our laboratory previously and propagated in BSR cells. Another rRABV rHEP-dG, carrying another G gene between G and L, was rescued previously [13]. CPV-S5 (CPV-2a) was propagated in CRFK cells. Challenge virus standard 24 (CVS-24) was propagated in suckling mice brains. Antibodies against RABV G and CPV VP2 were stored in our laboratory. Fluorescein isothiocyanate (FITC)-conjugate antibodies against the RABV N protein were purchased from Fujirabio Inc. (Malvern, PA, USA). FITC-conjugate antibodies against mouse IgG were purchased from Santa Cruz Biotechnology. Female Kunming (KM) mice were purchased from the Center for Laboratory Animal Science, Southern Medical University (Guangzhou, China) and housed in the animal facility of the College of Veterinary Medicine, South China Agricultural University. All animal experiments were carried out under the national standard Laboratory Animal Requirements of Environment and Housing Facilities (CALAS, GB 14925-2001) as well as following the National Institutes of Health guide for the care and use of Laboratory animals.

2.2. Virus propagation and titration

All rRABVs and parent strain HEP-Flury were propagated in BSR cells at 37 °C with 5% CO_2. Virus titration was determined by direct fluorescent antibody assay (dFA) in BSR cells as described previously [7]. Briefly, BSR cells in 96-well plates were inoculated with serial 10-fold dilutions of virus in DMEM and incubated at 37 °C for 4 days. The culture medium was discarded and cells were fixed with 80% acetone for 30 min at −20 °C. The cells were washed three times with phosphate buffer solution (PBS) and stained with FITC-labeled anti-RABV N antibodies (Fujirabio). After inoculation at 37 °C for 60 min, antigen-positive foci were counted under a fluorescence microscope (AMG, USA) and virus titers were calculated as focus forming units/ml (FFU/ml) using Carber's method.

2.3. Confirmation of the propagated viruses by RT-PCR and sequencing

The propagated viruses were confirmed by RT-PCR with four pairs of primers [13,29]. The primers N1 (sense) (5′-AGTCTCTA TAGGTTGAGC-3′) and N2 (antisense) (5′-GATGAAATAAGAGT GAGG-3′), both positioned on the N gene were used to amplify the RABV N gene (424 bp). RABVs carrying another G gene were confirmed with primers dG1 (sense) (5′-AAAGGGTGTTTGA GAGTTGG-3′), based on the first G gene sequence, and dG2 (antisense) (5′-ACAGGTTGGTACATCCTTCGTCC-3′), based on the second G gene sequence (744 bp). Primers GVP1 (sense) (5′-TTAGTCAA GAAAAGAGAGGAGTGTC-3′), based on the G gene sequence, and GVP2 (antisense) (5′-TACCCGTAGAAATCCC-3′), based on the VP2 gene sequence were used to amplify part of the G gene and part of the VP2 gene (822 bp). Primers VP2L1 (sense) (5′-AACTACCACAACAGGAG-3′), based on the VP2 gene sequence, and VP2L2 (antisense) (5′-TCTGTCAAAGTCATCCGATAAGGTC-3′), based on the L gene sequence, were used to amplify part of the VP2 gene and part of the L gene (794 bp). After viruses were harvested, total RNA was extracted using the E.Z.N.A™. Total RNA Kit II (OMEGA, USA) according to the manufacturer's instructions. Reverse transcription (RT) was performed using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Germany) according to the manufacturer's protocol. Amplification was carried out with rTaq Enzyme (TaKaRa, Japan) following the manufacturer's instructions. cDNA was sequenced (Sangon, Shanghai) to confirmed the propagated virus.

2.4. Western blotting

To confirm the expression of RABV G protein and VP2, western blotting was conducted as described previously [30]. Briefly, BSR cells were infected with rHEP-Flury, rHEP-VP2 and rHEP-dG-VP2 at a multiplicity of infection (MOI) of 0.1 FFU and inoculated at 37 °C. After 48 h, cells culture medium was discarded and cells were washed three times with cold PBS and then lysed on ice with RIPA buffer (Thermo Scientific). Total proteins were harvested and the target protein was separated by 12% SDS-PAGE, transferred on to polyvinylidene difluoride (PVDF) membranes and incubated with antibodies against RABV G or VP2. After incubation with goat anti-mouse antibody labeled with horseradish peroxidase (Vazyme, China), the PVDF membranes were stained with BeyoECL Plus A and B (Beyotime, China) according to the manufacturer's instructions. Protein fingerprints were shown using Fine-do x6 (Tanon, China).

2.5. Indirect immunofluorescence assay

Indirect immunofluorescence assay (IFA) was performed to confirm the expression of VP2 in CRFK cells. Monolayers of CRFK cells were infected with RABV at an MOI of 0.1 FFU and incubated at 37 °C for 48 h. The cell culture medium was discarded and cells were fixed with ethanol and acetone (1:1) for 30 min at room temperature. Cells were washed three times with PBS and stained with anti-VP2 antibodies at 37 °C for 3 h. After a second incubation with