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Generation of a recombinant rabies Flury LEP virus carrying an additional G gene creates an improved seed virus for inactivated vaccine production

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Abstract
The rabies Flury Low Egg Passage virus (LEP) has been widely used as a seed virus to generate inactive vaccine. Here, we established a reverse genetic system for LEP and generated a recombinant LEP virus (rLEP-G) that carries two identical G genes. This recombinant virus showed similar properties to those of LEP with respect to *in vitro* growth, neurotropism index, and virulence in mice. rLEP-G produced 4.3-fold more G protein than did LEP in BHK-21 cells. The inactivated vaccine generated from rLEP-G induced significantly higher virus neutralization titers in mice and dogs than those produced in response to LEP-derived vaccine. Our results suggest that rLEP-G is an improved seed virus candidate for inactivated rabies virus vaccine manufacture.

Keywords: Rabies virus, LEP, recombinant, inactivated vaccine

Introduction
Rabies virus (RV) belongs to the genus Lyssavirus of the family Rhabdoviridae, and causes a fatal neurological disease in humans and animals [1]. More than 55,000 people die of rabies each year, and about 95% of these deaths occur in Asia and Africa [2]. An estimated 31,000 people die from dog rabies in Asia each year, with most cases occurring in India and China [3,4]. The most cost-effective strategy for preventing rabies in people is to eliminate rabies in dogs via vaccination [5-7]. Inactivated rabies vaccine has been shown to be a safe and efficient means to control rabies in dogs. However, the vaccination rate of dogs in many developing countries is low, especially in rural areas, mainly due to low economic development and the high cost of vaccination [8]. More efficient and lower cost inactivated vaccine is, therefore, still needed.

The surface glycoprotein (G) of RV is the major antigen responsible for the induction of protective immunity [1]. Increasing G protein levels should, therefore, enhance the protective viral neutralization antibody (VNA) response. The rabies Flury low egg passage virus (LEP) has been widely used as a seed virus to generate inactive vaccine for humans and animals because of its high immunogenicity and high growth titer in cell culture [9]. Here, we generated a recombinant LEP virus that carries two identical G genes to increase G protein expression. Growth curves, neurotropism index, virulence, and the G protein expression level of the double-G LEP were tested *in vitro* and *in vivo*. The immunogenicity of the inactivated vaccine derived from this double-G LEP was also evaluated in mice and dogs and compared with that of LEP.

Materials and Methods
Viruses and cells
Neuroblastoma (NA) cells of A/J mouse origin were grown in Eagle’s minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). Baby hamster kidney (BHK-21) cells were grown in...
Dulbecco’s modified Eagle’s MEM (DMEM) supplemented with 10% FBS. The RV LEP (AV2012) was obtained from the China Veterinary Culture Collection Center and propagated in BHK-21 cells. A street virus, GX/09, was isolated from the brain of a dog that died of rabies and was propagated in the brain of adult mice. All viruses were kept in at -70°C before use.

**Plasmids construction**

Viral RNA was extracted with an RNeasy mini kit according to the manufacturer’s instructions (QIAGEN, Valencia, CA). The extracted RNA was subjected to RT-PCR with virus specific primer pairs (Table 1) and high-fidelity Pfx DNA polymerase (Invitrogen Corp., Carlsbad, CA) to generate three overlapping PCR fragments (F1, F2, and F3) that encompassed the entire viral genome. The assembled cDNA, containing the hammerhead ribozyme sequence (HamRz), was inserted between the Nhe I and Sma I sites of pCI. A Pme I restriction site was introduced into the G-L noncoding region by changing three nucleotide residues at positions 4907 (T to G), 4910 (G to T) and 4912 (C to A) by using a site-directed mutagenesis system (Invitrogen) with the primers shown in Table 1. The resultant plasmid was designated as pLEP. The cDNA of LEP strain genome in the anti-genomic orientation, and the hepatitis delta virus ribozyme sequence (HdvRz), was inserted between the Nhe I and Pme I, and the supernatants from virus-positive cultures were used to produce virus stocks in BHK-21 cells. The rescued virus generated from the full-length plasmid pLEP-G was designated as rLEP-G.

**Multistep growth assays**

Confluent BHK-21 or NA cell monolayers grown in 6-well plates were infected with LEP or rLEP-G at a multiplicity of infection (MOI) of 0.01. After incubation for 1 h at 37°C, the inoculums were removed and the cells were washed twice with PBS. BHK-21 and NA cells were replenished with DMEM containing 2% FBS or with MEM containing 0.2% FBS, respectively, and incubated at 37°C. Culture supernatant (100 μl) was harvested at 0, 1, 2, 3, 4, and 5 days post-inoculation for virus titration.

**Virus titration**

Monolayers of NA or BHK-21 cells in 24-well plates were infected with 10-fold dilutions of virus suspension and incubated at 37°C. At 48 h post-infection, an IFA test was performed. Foci were counted under a fluorescence microscope and calculated as focus forming unit/ml (FFU/ml).

The *in vitro* neurotropism index was expressed as the logarithm of the titer of virus stock in NA cells minus the logarithm of the titer of the same stock in BHK-21 cells [10]. The virus titers for neurotropism index calculation were based on the titration results of virus stock harvested at 72 h post infection.

**Western blotting**

BHK-21 cells grown in 6-well plates were infected with LEP or rLEP-G at an MOI of 1 and incubated for 48 h. G gene expression was confirmed by using western blotting as described previously [11]. Briefly, cell extracts were subjected to 10% polyacrylamide gel electrophoresis (SDS-PAGE), and then blotted to nitrocellulose membrane. The membrane was incubated with a mixture of mouse anti-G protein polyclonal antiserum and anti-β-actin monoclonal antibody (Santa Cruz Biotechnology), as the first antibody, and then with IRDye700-
Table 1 Primers used to construct the full-length cDNA clone and helper plasmids for Flury LEP

<table>
<thead>
<tr>
<th>Purpose</th>
<th>Name</th>
<th>Primer (5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>For F1 amplification</td>
<td>F1-F</td>
<td>TGGCGTAGCTGT7AAGCCGT7GAG7CCGTGAAGGAAACTTATAGTATAGTACGTTAACAACAAACAAAGAA&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>F1-R</td>
<td>GCCACGGGTACTCCACATATACTTGGTTGCT</td>
</tr>
<tr>
<td>For F2 amplification</td>
<td>F2-F</td>
<td>AGGCTGTATAAGTCTTTAAGGGAGCA</td>
</tr>
<tr>
<td></td>
<td>F2-R</td>
<td>ATCGGGGTCCCCGCTTCCACAAAC</td>
</tr>
<tr>
<td>For F3 amplification</td>
<td>F3-F</td>
<td>TATGGCTAGCTCTTGTTAAGGCTCCACGAATC</td>
</tr>
<tr>
<td></td>
<td>F3-R</td>
<td>CGATCCCCGGGGGGGGGGGCCCCGGGCCCCTCTTCCTTCCTTCCTTGGTTTACCTCTCCTTGGTTTACCTCTCCTTGGTTTACCTCTCCTTGGTTTACCTCT</td>
</tr>
<tr>
<td>For Pme I mutation</td>
<td>Prmu-F</td>
<td>GACTTAGTTTAACAGGATGACCGGGCC&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Prmu-R</td>
<td>GGCCGGTGATCCCTGTTTAAACTTCAAGTC</td>
</tr>
<tr>
<td>For G gene amplification</td>
<td>G1</td>
<td>ATGCGTTAAAACAGGGATGACTGAGATGACCGGGCC&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>G2</td>
<td>GCAATGTTAAAACAGGGATGACTGAGATGACCGGGCC&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>For N gene amplification</td>
<td>HN-F</td>
<td>GGCAGAATTCATGGATGCCGACAAAGATTGT</td>
</tr>
<tr>
<td></td>
<td>HN-R</td>
<td>CGCGGTACACTGATGACTGACACTCGAAATACG</td>
</tr>
<tr>
<td>For L gene amplification</td>
<td>HL-F</td>
<td>GGCAGAATTCATGGATGCCGACAAAGATTGT</td>
</tr>
<tr>
<td></td>
<td>HL-R</td>
<td>CGCGGTACACTGATGACTGACACTCGAAATACG</td>
</tr>
<tr>
<td>For LEP-G confirmation</td>
<td>G3</td>
<td>ATGCGTTTCTCTTGAATGTG</td>
</tr>
<tr>
<td></td>
<td>G4</td>
<td>GGGTTGAAAAAGCATATAC</td>
</tr>
</tbody>
</table>

<sup>a</sup> Restriction enzyme sites are shown in boldface. <sup>b</sup> The HamRz sequence is shown in italics. <sup>c</sup> The HdvRz sequence is shown in lowercase. <sup>d</sup> The nucleotides that were changed are underlined.
This page contains scientific text discussing the preparation and characterization of recombinant inactivated vaccines. The text describes the process of infection, preparation of inactivated vaccines, and the evaluation of their effectiveness. The text includes mentions of specific experiments, such as the titration of vaccines, neutralizing antibody assays, and the use of Western blot analysis for protein detection. The text also references the use of RT-PCR for amplification of viral RNA and the recovery of recombinant viruses from cloned cDNA. The page includes a diagram illustrating the schematic representation of recombinant virus containing two homologous G genes, which helps in visualizing the structure and organization of the viral genome. The text is part of a larger scientific paper discussing the development of anti-RV (rotavirus) vaccines.
indicating an approximate 4.3-fold increase in G protein levels in rLEP-G-infected cells due to expression from the additional G gene.

**Growth properties of rLEP-G in vitro**

To examine whether insertion an identical G gene into the genome of the LEP strain would affect the growth properties of the virus in vitro, we compared the growth of rLEP-G and LEP in both neuronal NA cells and nonneuronal BHK-21 cells. The multistep growth curves of rLEP-G (the third passage generation from BHK-21 cells) were compared with that of LEP in NA cells. There were no significant differences in growth patterns in both cell lines (Figure 3A, B) between rLEP-G and LEP. Furthermore, LEP and rLEP-G showed similar in vitro neurotropism indices of 0.84 and 0.85, respectively, which indicates that the additional G gene did not affect the in vitro neurotropism of the LEP strain.

To examine the genome stability of rLEP-G in cell culture, the virus was serially passed in BHK-21 cells 10 times. RT-PCR analysis revealed that the additional G gene in the rLEP-G genome was stable for at least 10 passages in BHK-21 cells. The titer of rLEP-G also remained high at approximately $1 \times 10^8$ FFU/ml, from the third to the tenth passage (Figure 4).

**Pathogenicity of rLEP-G in adult mice**

The G protein plays an important role in the pathogenicity of RV. Therefore, we needed to investigate whether insertion of an additional G gene would affect virulence. Accordingly, adult mice were inoculated with rLEP-G and LEP, respectively. The results showed that both rLEP-G and LEP caused neurological symptoms, such as paralysis and hyperactivity, and killed mice after i.c. inoculation. The LD$_{50}$ of rLEP-G following i.c. inoculation was 2 FFU, which was 2-fold higher than that of LEP (Figure 5). Peripheral pathogenicity was also examined following i.m. inoculation. All mice survived i.m. inoculation with $10^6$ FFU of rLEP-G, whereas, one of the five mice inoculated i.m. with the same dose of LEP died. This result indicates that the in vivo neuro-invasiveness and peripheral pathogenicity of LEP did not increase after insertion of the additional G gene.

**Induction of VNA in mice and dogs**

VNA is mainly induced by viral antigen G protein and plays a key role in immune protection from rabies. To investigate whether increased expression of G in the seed virus improves the VNA response of inactivated rabies vaccine, two inactivated vaccines were respectively preparations from LEP and rLEP-G infected cell cultures as described in materials and methods. The virus titers in supernatant of LEP and rLEP-G infected cell cultures were $10^{7.3}$ FFU/ml and $10^{7.4}$ FFU/ml respectively. The preparations were then tested in adult mice by using i.m.
inoculation with. There were no significant differences in VNA response between the mice that received 0.1 ml of 20-fold diluted LEP vaccine preparation (containing $5.2 \log_{10}$ FFU/0.1 ml) and those that received 0.1 ml of 20-fold diluted LEP-G vaccine preparation (for LEP and $5.1 \log_{10}$ FFU/0.1 ml for rLEP-G). However, the VNA of mice immunized with an 80-fold diluted rLEP-G vaccine preparation (containing $4.5 \log_{10}$ FFU/0.1 ml) was significantly higher than that of mice immunized with a similarly diluted LEP vaccine preparation (containing $4.4 \log_{10}$ FFU/0.1 ml) ($P < 0.01$) (Figure 6A). In dogs, 1 ml of rLEP-G vaccine preparation (containing $7.3 \log_{10}$ FFU/ml) induced a VNA mean titer of 54 IU, which was significantly higher than mean titer of 15 IU that induced by 1 ml of LEP inactivated vaccine (containing $7.2 \log_{10}$ FFU/ml) ($P < 0.05$) (Figure 6B). These results indicate that the rLEP-G strain is a more immunogenic seed virus for use in the development of inactive vaccines against rabies.

Discussion

Mass vaccination of dogs has proven to be an effective way to control rabies in this species. However, current live attenuated vaccines can have safety issues [14,15], and the use of current inactivated vaccines is limited in developing countries because of high manufacturing costs. Increasing G protein expression in RV seed viruses is hypothesized to improve rabies inactivated vaccines not only via immunogenicity but also through increased manufacturing productivity. Previous studies have demonstrated the feasibility of introducing an additional G gene into the RV genome [16-18]. By using reverse genetics technology, Faber et al. generated a double-G gene recombinant RV (SPBNGA-GA). This double-G virus showed a 1.6-fold increase in G protein expression in cell culture relative to that of its single-G gene parent virus (SPBNGA). Inoculation with SPBNGA-GA live virus provided more efficient protective immunity than did the SPBNGA strain in mice. However, the utility of SPBNGA-GA as a seed virus for inactivated vaccine production has not been evaluated further [18]. In another study, Hosokawa-Muto et al. generated a double-G gene recombinant RV RC-HL. This virus showed a 1.5-fold increase in G protein expression in cell culture compared with that induced in the single-G gene parent virus RC-HL [16]. When the recombinant double-G gene recombinant RV RC-HL and wild-type RC-HL were inactivated and used to vaccinated mice, no significant difference in RV neutralization antibody responses was observed.

Here, we generated a recombinant LEP virus carrying an additional G gene, rLEP-G. This recombinant virus produced strikingly higher levels of G protein in cell culture and showed similar in vitro growth properties and bio-safety characteristics. Inactivated vaccine prepared from rLEP-G induced significant higher RV VNA titers in mice and dogs than those induced by LEP-derived vaccine, indicating that rLEP-G represents an improved seed virus candidate for inactivated RV vaccine manufacture.

Since G protein is a major contributor to the pathogenicity of rabies virus [19-22], we evaluated whether this increase in G protein expression affected the virulence of the rLEP-G virus. We found that the insertion of this additional G gene results in increase of G protein expression but not in increase of the neuro-invasiveness or peripheral pathogenicity of the virus in adult mice. In other studies, introduction of one or two additional G genes into the genome of rabies virus resulted in a higher level of G protein expression and attenuate pathogenicity in adult mice [17,18,23]. As the pathogenicity of a particular RV strain correlates inversely with its ability to trigger apoptosis in neuron cells [23], one possible explanation for this discrepancy is that some lethal viruses, such as LEP and rLEP-G, rarely induced apoptosis in neuron cells. In fact, our previous data had shown that there was no clear correlation between apoptosis

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**Figure 5** Pathogenicity of LEP and rLEP-G in adult mice. Six-week-old Balb/c mice were inoculated intracerebrally with LEP (A) or rLEP-G (B) in a 30 μl volume at doses of $10^0$ to $10^3$ FFU (black inverted triangle: $10^0$ FFU, black triangle: $10^1$ FFU, black square: $10^2$ FFU, black dot: $10^3$ FFU). After infection, mice were observed for 21 days for clinical signs or death. The MLD50 of each virus was calculated by using the method of Reed and Muench.

**Figure 6** VNA titers in mice and dogs immunized with inactivated LEP or rLEP-G vaccine preparations. (A) Groups of 10 mice were immunized with 20- or 80-fold dilutions of inactivated vaccine preparations of LEP or rLEP-G. (B) Groups of 6 dogs were immunized with 1 ml of inactivated vaccine preparations of LEP or rLEP-G. Blood samples were collected at 3 weeks post-vaccination and subjected to the VNA titration test. VNA were determined by using the rapid fluorescence inhibition test. Titers were normalized to international units by using the World Health Organization standard. **, $P < 0.01$; *, $P < 0.05$. 

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induction and the viral replication titers or glycoprotein expression level among LEP and its relative mutants or chimerical viruses[19].

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Authors’ contributions
LT, HZ and TH carried out the plasmid construct cloning, rescued and prepared the manuscript. CY participated in experiment design and prepared the manuscript. All authors have read and approved the submitted manuscript.

Competing interests
The authors declare that they have no competing interests.

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