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Genetically modified rabies virus-vectored Ebola virus disease vaccines are safe and induce efficacious immune responses in mice and dogs

Lei Shuai, Xijun Wang, Zhiyuan Wen, Jinying Ge, Jinliang Wang, Dandan Zhao, Zhigao Bu*

Key Laboratory of Veterinary Public Health of Ministry of Agriculture, State Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Harbin, 150001, China

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ABSTRACT

Ebola viruses (EBOVs) are zoonotic pathogens that cause EBOV disease (EVD) with high case fatality in humans. Currently, EVD vaccines are still under development in several countries. Here, we generated two recombinant rabies viruses (RABVs), rERAG$_{333E}$/ZGP and rERAG$_{333E}$/SGP, expressing the Zaire EBOV glycoprotein (ZGP) or Sudan EBOV glycoprotein (SGP) gene based on a modified ERA vaccine strain (rERAG$_{333E}$) vector platform. The recombinant RABVs retained growth properties similar to those of the vector virus in BSR cell culture and efficiently expressed ZGP or SGP. After intracerebral (*i.c.*) inoculation with rERAG$_{333E}$/ZGP or rERAG$_{333E}$/SGP, all adult mice showed no signs of disease or weight loss and suckling mice maintained similar survivorship curve as those mice inoculated with control vector rERAG$_{333E}$, demonstrating that ZGP or SGP expression did not increase the virulence of the vector. Mouse immunization studies showed that vaccination with rERAG$_{333E}$/ZGP and rERAG$_{333E}$/SGP induced Zaire or Sudan EBOV neutralizing antibody (VNA) responses and IgG, IgG2a responses to ZGP or SGP, suggesting their potential as oral or inactivated bivalent vaccines against rabies and EVD. Most importantly, all dogs immunized orally with rERAG$_{333E}$/ZGP developed long-lasting ZEBOV and RABV VNA responses with or without previous rabies vaccine immunization history. Live rERAG$_{333E}$ with EBOV GP thus appear to have the potential to be oral vaccines for free-roaming animals in endemic areas of EVD and rabies, and may serve as inactivated vaccines for use in humans.

1. Introduction

Ebola virus (EBOV) disease (EVD), is a serious zoonotic disease due to its high-case fatality rate, potential for human-to-human and hospital-based transmission, no effective specific treatment and challenges in identification of transmission routes, and the natural reservoir of the virus (Kinsman, 2012; Negredo et al., 2011; Sullivan et al., 2003). The recent 2014 extensive outbreak of EVD in West Africa already surpassed the number of confirmed cases from the previous outbreaks combined. Unfortunately, the natural reservoir host of EBOV remains unknown. Of particular concern, EBOV is believed to be animal-borne and it is assumed that bats are the most likely reservoir (WHO, 2015). In addition, free-roaming dogs and pigs have been found to be infected EBOV and have potential to become endemic and cause zoonotic transmission to humans and non-human primates (NHPs) (Weingartl et al., 2013). With lack of effective approach for prevention of EVD, consequently, the development of efficacious oral vaccines to cut off the transmission from free-roaming domestic animals and wild animals has always been of utmost importance and urgency.

EBOV glycoprotein (GP), dispersed throughout the viral envelope as trimeric spikes, could function in attachment and entry of virus, cell rounding, cytotoxicity, down-regulation of host surface proteins, and enhancement of virus assembly and budding (Chandran et al., 2005; Dolnik et al., 2004; Falzarano et al., 2006; Manicassamy et al., 2005; Takada and Kawaoka, 2001). Moreover, GP is expressed based on vector platform of adenovirus or vesicular stomatitis virus (VSV) that is immunogenic and effective in many animal models against EBOV hemorrhagic fever (Geisbert et al., 2008; Geisbert and Feldmann, 2011; Manicassamy et al., 2005; Marzi et al., 2011).

* Corresponding author. 678 Haping Road, Xiangfang District, Harbin, Heilongjiang, 150069, China.
E-mail address: buzhigao@caas.cn (Z. Bu).

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Rabies, a major public health problem around the world, is responsible for about 60,000 human deaths per year worldwide, and about 40% are children who become infected by rabies-infected dogs in Africa and Asia (WHO, 2013). Currently, attenuated RABV strains are routinely used worldwide as oral vaccines in dogs and other carnivores (Bankovskiy et al., 2008; Cliquet et al., 2007; Rosatte et al., 2009). Moreover, RABV vaccines have been proven to be excellent live vaccine vectors and were widely used as viral vector to develop bivalent live vaccines (Blaney et al., 2011; Faul et al., 2009; Kurup et al., 2015).

RABV GP is a potent immunogen and an important virulence factor (Ito et al., 2001; Morimoto et al., 2000). Meanwhile, the mutation from arginine or lysine to other amino acid at residue 333 of glycoprotein (G_{333}) in wild-type RABV strains can reduce or eliminate the virulence of RABV (Faber et al., 2005). Hence, we previously developed a candidate oral rabies vaccine ($rERAG_{333E}$), which is safe and induces long-lasting protective immune response against RABV when administered orally in mice and dogs (Shuai et al., 2015). Accordingly, we perform the first experiments using $rERAG_{333E}$, as a live vector platform expressing the wild-type *GP* gene of Sudan EBOV (*SGP*) or Zaire EBOV (*ZGP*). The feasibility of the recombinant RABVs to serve as bivalent oral vaccines against rabies and EVD in mice and dogs was evaluated.

2. Materials and methods

2.1. Cells and viruses

BSR and 293 cells were grown in Dulbecco's Modified Eagle's Minimal Essential Medium (DMEM, Gibco) supplemented with 5% or 10% fetal bovine serum (FBS, ExCell). The recombinant viruses, rERA-eGFP, $rERAG_{333E}$, rVSV-ZGP and rVSV-SGP, were generated. A previously described VSVΔG-eGFP pseudovirus system (Wang et al., 2006) was adapted and modified to construct VSVΔG*GFP/ZGP and VSVΔG*GFP/SGP. All viruses were stored at −70 °C before use.

2.2. Plasmid construction and virus rescue

Chemically synthesized *ZGP* (GenBank accession No. NC_002549.1) and *SGP* (GenBank accession No. NC_006432.1) were amplified with paired primers *ZGPF/ZGPR* or *SGPF/SGPR* (Table 1), and cloned into the *Pme* I site of $rERAG_{333E}$ (Shuai et al., 2015). The resulting full-length plasmids were designated as pCI-$rERAG_{333E}$/ZGP or pCI-$rERAG_{333E}$/SGP.

The protocols for the recombinant RABVs rescue were described previously (Shuai et al., 2015; Tao et al., 2010, 2011). Rescued viruses were confirmed by using reverse transcription (RT)-PCR, indirect immunofluorescence assay (IFA) (Wang et al., 2012) and western blotting (Ge et al., 2007). The rescued viruses were designated as $rERAG_{333E}$/ZGP and $rERAG_{333E}$/SGP, respectively.

Table 1
Primers used for construction of the cDNA of wild-type *GP* gene of ZEBOV or SEBOV.

Name	Primers (5′-3′)[a]
ZEGF	CAG**GTTTAAAC**ATGAAAAAAACAGGCAACACCACTGCCGCCACC ATGGGCGTTACAGGAATA
ZEGR	GCCG**GTTTAAAC**TTAAAAGACAAATTTGC
SEGF	ATG**GTTTAAAC**ATGAAAAAAACAGGCAACACCACTGCCGCCACC ATGGGGGGTCTTAGCCTAC
SEGR	GCCG**GTTTAAAC**TTAGCAAAGCAGCTTGCAAACGC

[a] Restriction enzyme site *Pme* I is shown in bold. The Kozak sequence is underlined.

2.3. Genetic stability and growth properties assays

To evaluate the genetic stability of the inserted genes of recombinant RABVs, the viruses were serially passaged 20 times in BSR cells. The viruses in different passages were examined by RT-PCR and sequences of the ZGP or SGP gene fragment with primers NXF (5′-GATCCTCAGGGATACTCTTG-3′) and NXR (5′-AGCCACAAGTCATCGTCATC-3′). To examine the growth properties of viruses, 80% confluent BSR cell monolayers grown in 6-well plates were infected with recombinant RABVs or vector at a MOI of 0.01. After incubation for 1 h at 37 °C, the inoculums were removed and the cells were washed twice with PBS. BSR cells were then replenished with DMEM containing 2% FBS, and incubated at 37 °C. Viruses were harvested at 24-h intervals for 144 h post-inoculation for virus titration, and virus titers were determined in BSR cells and expressed as focus-forming units (FFU)/mL by IFAs.

2.4. Inactivation of viruses

The supernatants of recombinant RABVs passaged in BSR cells were transferred to a microcentrifuge tube and spun for 10 min at 10,000 g to remove cell debris. The virus suspensions, titrated in BSR cells, were then inactivated with 0.03% β-propiolactone (BPL) overnight at 4 °C. After hydrolysis of BPL for 1 h at 37 °C, the inactivated viruses were examined for absence of cytopathogenicity for BPL and lack of live RABV by IFA during each three passages into BSR cells, respectively. Then, inactivated viruses were mixed with 25% (v/v) Rehydraphos AlPO4 adjuvant (Reheis, Berkeley Heights, NJ) respectively, and stored at 4 °C for vaccination.

2.5. Animal studies and laboratory facility

All animals were handled in strict accordance with recommendations described in the Guide for the Care and Use of Laboratory Animals (Council, 2011). All animal studies were approved by the Institutional Animal Care and Ethics Committee (IACEC) of Harbin Veterinary Research Institute (HVRI), Chinese Academy of Agricultural Sciences (CAAS). All procedures were carried out by trained personnel in a biosafety level 3 (BSL-3) facility at HVRI, CAAS. BALB/c mice (Vital River, Beijing, China) and beagles (Guangzhou General Pharmaceutical Research Institute, Guangzhou, China) were housed in cages, under controlled conditions of humidity, temperature, and light (12-h light/12-h dark cycles). Food and water were available *ad libitum*.

2.6. Pathogenicity experiments

Groups of ten 6-week-old female BALB/c mice were incubated intracerebrally (*i.c.*) with 0.03 mL PBS (mock infection control) or PBS containing 10^6 FFU virus, respectively. The mice were observed daily for signs of disease, bodyweight changes or death for 3 weeks. Meanwhile, litters of three-day-old BALB/c suckling mice were incubated *i.c.* with 0.03 mL PBS or PBS containing 10^6 FFU or 10^4 FFU viruses, respectively. The suckling mice were monitored daily for clinical signs of encephalitis after infection, and moribund mice were humanely euthanized. Survival of suckling mice were monitored daily for 21 days, and survival rates obtained with the different infection groups were compared.

2.7. Immunization studies in mice

Groups of ten 6-week-old female BALB/c mice were vaccinated orally with 0.1 mL PBS containing $10^{6.5}$ FFU recombinant RABVs or vector virus once by instilling into the buccal cavity with a micro