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Short communication

Controlled viral glycoprotein expression as a safety feature in a bivalent rabies-ebola vaccine

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ABSTRACT

Using a recombinant rabies (RABV) vaccine platform, we have developed several safe and effective vaccines. Most recently, we have developed a RABV-based ebolavirus (EBOV) vaccine that is efficacious in nonhuman primates. One safety feature of this vaccine is the utilization of a live but replication-deficient RABV construct. In this construct, the RABV glycoprotein (G) has been deleted from the genome, requiring G trans complementation in order for new infectious viruses to be released from the initial infected cell. Here we analyze this safety feature of the bivalent RABV-based EBOV vaccine comprised of the G-deleted RABV backbone expressing EBOV glycoprotein (GP). We found that, while the level of RABV genome in infected cells is equivalent regardless of G supplementation, the production of infectious virus is indeed restricted by the lack of G, and most importantly, that the presence of EBOV GP does not substitute for G. These findings further support the safety profile of this replication-deficient RABV–EBOV bivalent vaccine.

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Rabies and Ebola are viruses of the order Mononegavirales, being single-stranded negative sense RNA viruses. Rabies virus, of the family *Rhabdoviridae*, genus *Lyssavirus*, consists of five genes, which encode for the membrane-associated proteins: matrix (M) and glycoprotein (G); and the structural proteins: nucleoprotein (N), phosophoprotein (P) and polymerase (L) (Conzelmann et al., 1990; Tordo et al., 1986). Ebola virus, of the family *Filoviridae*, consists of seven genes, which encode for the membrane-associated proteins: matrix (VP40), minor matrix (VP24) and glycoprotein (GP); and the structural proteins: minor nucleoprotein (VP30), nucleoprotein (NP), polymerase cofactor (VP35) and polymerase (L) (Hartlieb and Weissenhorn, 2006; Stahelin, 2014).

The viral glycoproteins have similar functions for the members of the Mononegavirales. Rabies virus (RABV) G facilitates entry into permissive cells, enables cell-to-cell spread of the virus, and supports budding of the virion from the host cell membrane (Etessami et al., 2000; Mebatsion et al., 1996a; Pulmanausahakul et al., 2008; Schnell et al., 2010). Similarly, EBOV GP is necessary for virion entry and fusion to release the viral capsid (Hunt et al., 2012; Marzi et al., 2006; Nanbo et al., 2010; Takada, 2012). Both RABV G and EBOV GP are highly immunogenic, and antibodies generated against these proteins can neutralize virus and block infection (Blaney et al., 2013; Faber et al., 2002; Marzi et al., 2013). RABV neutralizing antibodies toward G are the host's primary defense against the invading pathogen (Goudsmit et al., 2006; Schnell et al., 2010), and pre-exposure vaccination is effective in preventing an otherwise potentially lethal disease (Manning et al., 2008). Moreover, the passive application of antibodies against RABV G in combination with an active immunization schedule with killed RABV virions successfully prevents rabies after infection when administered appropriately (McGettigan, 2010). Recent results indicate that, during EBOV infection, CD4+ and CD8+ T cells had a minimal role in providing protection, while anti-GP antibodies induced by the vaccine appeared to be critical for protecting the animals (Marzi et al., 2013).

While Ebola is not a widespread or chronic disease, its pathogenicity, virulence and transmission have generated interest in a vaccine for military and biodefense purposes (Kuhn et al., 2011; Richardson et al., 2010). The current outbreak in West Africa has highlighted the need for an EBOV vaccine. RABV has been shown as an exceptional vaccine vector for multiple antigens including EBOV (Faber et al., 2005; Mebatsion et al., 1996b; Schnell et al., 1994; Smith et al., 2006), and RABV is also endemic in areas where EBOV is found. Therefore, a RABV virus that expresses the Ebola GP is an attractive vaccine candidate. Based on our previous research and findings concerning the importance of the elicitation of neutralizing antibodies for protection, the expression of G and GP in our bivalent vaccine is critical to its success (Blaney et al.,

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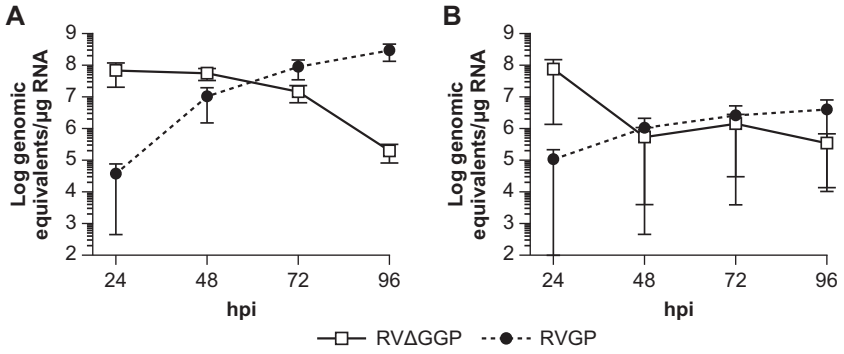

Fig. 1. Quantitative PCR to detect RABV nucleoprotein over time in a multi-step growth curve (MOI 0.01). Data points are log mean genomic equivalents per microgram of total RNA ($n = 2$) with SEM error bars. RVΔGGP, □; RVGP, ●; viral genome replication was not statistically different on (A) BSR-G and (B) VeroE6 cells for either virus ($n = 2$; Student's t test, $p = 0.35$ and 0.24 respectively).

2013; Faber et al., 2002; Marzi et al., 2006; McKenna et al., 2004). In a previous study, our G-deleted RABV expressing GP (RVΔG-GP] provided 50% protection against EBOV challenge in NHPs as compared to 100% protection conferred by the replication competent version of the vaccine. If RVΔG-GP can be optimized for immunogenicity to provide 100% protection from EBOV challenge, it would be an ideal vaccine choice based on safety and efficacy data.

Controlled growth is a key safety feature for the potential marketability of this live RABV vaccine. The growth of RVΔG-GP is controlled via an efficient on/off gene expression system. RVΔG-GP was recovered and grown in BSR cells (a hamster kidney cell (BHK) line expressing RABV G (BSR-G)), where G expression is regulated by a Tet-off reporter gene system (Blaney et al., 2011; Gomme et al., 2010). Whereas VeroE6 cells are approved for production of rabies vaccines (Barrett et al., 2009), it is anticipated that RVΔG-GP would be manufactured on a newly developed VeroE6 cell line expressing G via the same mechanism as BSR-G cells, provided that RVΔG-GP does not grow on VeroE6 alone.

Our goal in this study was to further elucidate the mechanism whereby viral growth is restricted. In so doing, we sought to ensure the safety of the vaccine during future large-scale production on VeroE6 cells, as we had concerns about the functions of residual G and the utilities of GP. Growth limitation of RVΔG-GP based on withholding RABV G has been demonstrated in vitro, and western blotting of virus proteins confirmed the absence of G and presence of GP for our construct (Blaney et al., 2011). Previous studies have shown that RVΔG-GP grows to similar titers as its replication-competent counterpart RVGP, when grown in BSR-G cells, but no infectious RABV is detected when VeroE6 cells are infected (Papaneri et al., 2012). We wanted to confirm the lack of growth of infectious virus on VeroE6 cells by multiple methods to answer questions that arose during the development of this vaccine, namely whether: (1) residual G in the supernatant from growth on BSR-G cells permits RVΔG-GP to replicate in VeroE6 cells; (2) RVΔG-GP grows on VeroE6 cells by substituting GP for G, as similar results have been shown for VSV (Schnell et al., 1998). Overall, we sought to determine if the safety profile of RVΔG-GP supports its further optimization and large-scale production for vaccine manufacture.

The recovery and propagation of the recombinant vaccine viruses used in this study have been described previously (Blaney et al., 2011; McGettigan et al., 2003; Papaneri et al., 2012). BSR cells were originally derived from BHK-21 cells; BSR-G cells stably express RABV G after stimulation with doxycycline (Gomme et al., 2010). VeroE6 cells were from ATCC (CRL-1586). Both cell lines were cultured in DMEM (Gibco) supplemented with 10% FBS (Hyclone).

We previously established that no infectious RVΔG-GP is found in supernatant from growth curves on VeroE6 cells via focus forming assay based on RABV N detection (Papaneri et al., 2012). To determine the capability of the virus to spread on VeroE6 cells by utilizing GP or residual G from stock preparation on BSR-G cells, we utilized qRT-PCR, confocal microscopy and electron microscopy to analyze RVΔG-GP passaged on VeroE6 and BSR-G cells.

Here, we demonstrate that RVΔG-GP viral genomic RNA can be detected via qPCR at similar levels as RVGP after direct infection of VeroE6 cells (Fig. 1). The fact that the level of RABV N genomic material present is comparable for RVΔG-GP on either BSR-G or VeroE6, yet the virus fails to spread from cell to cell in VeroE6, indicates a failure in the assembly or budding of the virion from the cell after entry. To emphasize that RVΔG-GP is not capable of regaining the ability to propagate in VeroE6, we performed a serial passage experiment in VeroE6 (Fig. 2). The data suggest that while the starting material RVΔG-GP for the growth curve was infectious, it was not able to propagate and by the third passage RABV N RNA was equal to the negative control. qRT-PCR was performed as follows. RNA was derived from infected cells at indicated timepoints using an RNeasy kit (Qiagen). Reverse transcription was performed using a Transcriptor High Fidelity cDNA Synthesis Kit (Roche) with primer RP381 (5-ACACCCCTACAATGGATGC-3) for RABV N synthesis. Real-time PCR was performed on an ABI7900HT Fast machine using a Dynamo Probe qPCR kit (ThermoScientific). Two replicates were performed in duplicate. The following primers and probes were used: RABV N RNA sense primer (5-AGAAGGGAATTGGGCTCTG-3), RABV N RNA antisense primer (5-TGTTTTGCCCGGATATTTTG-3), RABV N RNA probe (5-CGTCCTTAGTCGGTCTTCTCTTGAGTCTGT-3).

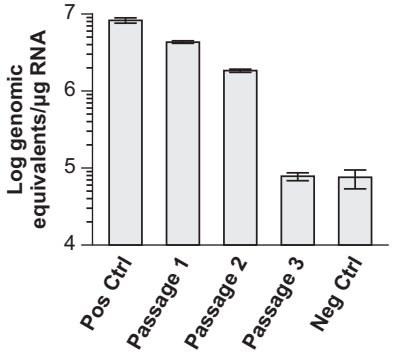

Fig. 2. Quantitative PCR to detect RABV nucleoprotein upon serial passage of RVΔGGP on VeroE6 cells. Viral supernatant was passaged at 48 h post-infection. Bars are log mean genomic equivalents per microgram of total RNA ($n = 2$) with SEM error bars. Positive control is RNA from the RVΔGGP stock used to infect for passage 1. Negative control is an irrelevant RNA.