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Controlled viral glycoprotein expression as a safety feature in a bivalent rabies-ebola 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 5 genes, which encode for the membrane-associated proteins: matrix (M) and glycoprotein (G); and the structural proteins: nucleoprotein (N), phosphoprotein (P) and polymerase (L) (4, 31). Ebola virus, of the family *Filoviridae*, consists of 7 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) (10, 29).

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 (5, 19, 23, 26). Similarly, EBOV GP is necessary for virion entry and fusion to release the viral capsid (11, 15, 21, 30). Both RABV G and EBOV GP are highly immunogenic, and antibodies generated against these proteins can neutralize virus and block infection (2, 7, 14). RABV neutralizing antibodies toward G are the host's primary defense against the invading pathogen (9, 26), and pre-exposure vaccination is effective in preventing an otherwise potentially lethal disease (13). 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 (16). 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 (14).

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 (12, 24). 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 (6, 20, 27, 28), 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

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neutralizing antibodies for protection, the expression of G and GP in our bivalent vaccine is critical to its success (2, 7, 15, 18). In a previous study, our G-deleted RABV expressing GP (RV G-GP) provided 50% protection against EBOV challenge in NHPs as compared to 100% protection conferred by the replication competent version of the vaccine. If RV G-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 RV G-GP is controlled via an efficient on/off gene expression system. RV G-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 (3, 8). Whereas VeroE6 cells are approved for production of rabies vaccines (1), it is anticipated that RV G-GP would be manufactured on a newly developed VeroE6 cell line expressing G via the same mechanism as BSR-G cells, provided that RV G-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 RV G-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 (3). Previous studies have shown that RV G-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 (22). 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 RV G-GP to replicate in VeroE6 cells; 2) RV G-GP grows on VeroE6 cells by substituting GP for G, as similar results have been shown for VSV (25). Overall, we sought to determine if the safety profile of RV G-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 (3, 17, 22). BSR cells were originally derived from BHK-21 cells; BSR-G cells stably express RABV G after stimulation with doxycycline (8). 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 RV G-GP is found in supernatant from growth curves on VeroE6 cells via focus forming assay based on RABV N detection (22). 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 RV G-GP passaged on VeroE6 and BSR-G cells.

Here, we demonstrate that RV G-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 RV G-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 RV G-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 RV G-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-CGTCCCTAGTCGGTCTTCTCTTGAGTCTGT-3). Custom primers and probes were purchased from Applied Biosystems.

To corroborate these results, RVGP and RV G-GP infected Vero E6 and BSR cells were analyzed at 48h post-infection via confocal microscopy. Expression of viral proteins RABV N, G, and EBOV GP was detected using a dual staining method for either RABV N and RABV G or RABV N and EBOV GP (Table 1 and Fig 2). Staining for all three antibodies at once was attempted; however, background fluorescence was increased, and image detail suffered. Therefore, the experiment was performed using the dual staining method as illustrated. Briefly, cells were seeded on glass coverlips in 12 well plates on day 0. On day 1, cells were infected at an MOI of 1 with either RVGP or RV G-GP using cellgro Complete serum free media (Mediatech) and incubated for 48h at 37°C/5%CO₂. Media was then removed, and wells were gently washed with PBS. Cells were fixed using 4% paraformaldehyde for 20min at RT. Fixed cells were washed 3X with PBS, then blocked and permeabilized in PBS with 1% BSA, 0.3M Glycine and 0.1% Triton X-100 for 1h at RT. Cells were washed 2X with PBS and labeled with primary antibody for RABV N, G or EBOV GP overnight at 4°C. Cells were washed 3X with PBS, and stained with secondary antibody for 1h at RT. Coverslips were then mounted on glass slides using Vectashield mounting media with DAPI (Vector Labs) and visualized using the Broadband Confocal Leica TCS SP5 II and Leica LAS AF software for analysis. RVGP and RV G-GP were found in both cell lines as indicated by the presence of RABV N. However, although G and GP were detected in both cell lines for RVGP, RABV G was not detected in RV G-GP infected VeroE6 cells (Fig 2, 1B). Of note, fluorescence of G present in the BSR-G cells alone is evident and distinguishable from the presence of G associated with the viral infection of the cell line. The absence of RABV G staining in RV G-GP infected VeroE6 cells confirms that RABV G is not synthesized and indicates that residual RABV G from the initial infection is not recycled and incorporated into new budding virions.

Electron microscopy further reinforces that RV G-GP is replication deficient and limited at the final steps of assembly and budding of infectious virions in VeroE6 cells (Fig 3). Thin-section electron microscopy was performed as follows. One-day-old 90% confluent T75 flasks of either BSR-G or VeroE6 cells were infected in duplicate at an MOI of 0.1 with

either RVGP or RV G-GP using cellgro Complete serum free media (Mediatech). Flasks were incubated at 37°C/5%CO₂ for 96h. Supernatant was removed, and adherent cells were fixed with 0.1% paraformaldehyde in Millonig's Sodium Phosphate Buffer for 10min at RT. Cells were washed with media and incubated with primary antibodies for glycoproteins (see Table 1) for 3h at RT followed by gold-conjugated secondary antibodies for 2h at RT. Cells were washed with media and fixed with 2.5% glutaraldehyde + 2% paraformaldehyde. Cells were scraped from the flasks and pelleted by centrifugation. Samples were kept for 24h at 4°C to complete fixation. Post-fixation, staining, embedding, and thin-sectioning were performed as described previously (3). The results are in agreement with previous findings that RABV virion formation and budding is hindered in G-deleted RABV mutants (19). Of RV GGP infected BSR-G cells, approximately 95% of all the extracellular and budding particles exhibited GP labeling only, lightly labeled with 15nm gold particles (Fig 3, B). Only 5% of the extracellular or budding particles exhibited labeling with both GP 15nm and G 5nm particles. RVGP labeled for both G and GP in both BSR-G and VeroE6 cells (Fig 3, A and C). Immunogold labeling was not performed for VeroE6 cells infected with RV G-GP, due to the tremendous reduction of budding or extracellular virus-like particles in our samples (Fig 3, D). In our RV G-GP -infected VeroE6 cells, pleomorphic shapes and VLPs were found in less than 5% of all cells examined. A few cells also exhibited areas of electron-dense material, which may have been an accumulation of viral-related proteins. No gold particles were found in negative control samples, where over 200 sections were analyzed per sample.

Evidently, RV G-GP is unable to utilize residual G to proliferate from VeroE6 cells, and the translated Ebola GP does not effectively substitute to permit continued viral particle formation or release. Hence, the vaccine is incapable of recombining to a replicative genotype and, furthermore, unable to revert to a pathogenic phenotype. These results bolster the safety profile of this replication-deficient RABV-EBOV bivalent vaccine. Further animal studies would be required to determine if an RV G-GP optimized for greater GP incorporation could provide 100% protection from EBOV challenge.

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Highlights

Our replication deficient rabies-ebolavirus bivalent vaccine candidate is capable of efficient infection in cell culture as evidenced by equivalent genome levels between the rabies virus glycoprotein trans-complementing cell line and target VeroE6 cells.

In our rabies G deleted vaccine construct, RABV-G is not synthesized nor is it recycled and incorporated into budding virions as determined by electron and confocal microscopy.

Figure 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. RV ΔGFP, □;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).

Figure 2.

Quantitative PCR to detect RABV nucleoprotein upon serial passage of RV GGP on VeroE6 cells. Viral supernatant was passaged at 48h 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 RV GGP stock used to infect for passage 1. Negative control is an irrelevant RNA.

Figure 3.

Confocal microscopy at 126X magnification using fluorescently labeled antibodies shows that RVG is not synthesized in RV Δ GGP-infected VeroE6 cells. BSR-G and VeroE6 cells were infected with RVGP or RV Δ GGP at an MOI of 0.01, and then fix and stained at 48hpi according to staining method in Table 1. Antibody set N/G is for RABV N and RABV G detection; N is red and G is green. Antibody set N/GP is for RABV N and EBOV GP detection; N is red and GP is green.

Figure 4.

Electron microscopy of VeroE6 and BSR-G cells infected with RV GGP and RVGP followed by immunogold labeling for RABV G and EBOV GP at 96hrs post infection. Cells were infected and prepared as described in the text. Gold-tagged antibodies confirm glycoprotein expression on virion surface. A) RVGP in BSR-G cells; B) RV GGP in BSR-G cells; C) RVGP in VeroE6 cells; D) RV GGP in VeroE6 cells (67900X). 96hpi @ 108000X unless noted. RVG labeled with 5nm particles. GP labeled with 15nm particles. Immunogold labeling of D was not performed due to lack of extracellular virions at sufficient quantities to justify staining.

Table 1

Confocal Staining Method. 4% Paraformaldehyde fixation, then blocked and permeabilized in PBS with 1% BSA, 0.3M Glycine and 0.1% Triton X-100.

Antibody set	N/G	N/GP
Primary	Mouse anti-RABV N (Millipore) and Rabbit anti-RABV G ¹	Mouse anti-RABV N (Millipore) and Human anti-EBOV GP ²
Secondary	anti-mouse594/anti-rabbit488 (AlexaFluor)	anti-mouse594/anti-human488 (AlexaFluor)

¹#1063, In-house polyclonal antibody from Matthias Schnell, Thomas Jefferson University.

²KZ52, monoclonal antibody kindly provided by D. Burton, The Scripps Research Institute.