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Further characterization of the immune response in mice to inactivated and live rabies vaccines expressing Ebola virus glycoprotein

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Abstract

We have previously developed (a) replication-competent, (b) replication-deficient, and (c) chemically inactivated rabies virus (RABV) vaccines expressing ebolavirus (EBOV) glycoprotein (GP) that induce humoral immunity against each virus and confer protection from both lethal RABV and mouse-adapted EBOV challenge in mice. Here, we expand our investigation of the immunogenic properties of these bivalent vaccines in mice. Both live and killed vaccines induced primary EBOV GP-specific T-cells and a robust recall response as measured by interferon- γ ELISPOT assay. In addition to cellular immunity, an effective filovirus vaccine will likely require a multivalent humoral immune response against multiple virus species. As a proof-of-principle experiment, we demonstrated that inactivated RV-GP could be formulated with another inactivated RABV vaccine expressing the nontoxic fragment of botulinum neurotoxin A heavy chain (HC50) without a reduction in immunity to each component. Finally, we demonstrated that humoral immunity to GP could be induced by immunization of mice with inactivated RV-GP in the presence of pre-existing immunity to RABV. The ability of these novel vaccines to induce strong humoral and cellular immunity indicates that they should be further evaluated in additional animal models of infection.

Keywords

Ebola virus; rabies virus; vaccine; T cell; multivalent; platform

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1. Introduction

Several viral species of the *Ebolavirus* genus and *Marburgvirus* genus, Family *Filoviridae*, cause severe and often fatal viral hemorrhagic fever in humans and nonhuman primates [1]. The search for a multivalent filovirus vaccine that confers protection from the Ebola virus (EBOV) and Marburg virus species of public health concern continues as no candidate is approaching licensure [2, 3]. The high case fatality rate, public health threat in Africa, and biodefense concerns associated with these viruses drive vaccine development. Several vaccination strategies have been developed over the past decade that confer protection in animal models but issues of safety, preexisting vector immunity, manufacturing, or a lack of commercial interest have slowed progress [2, 4–7]. Recent studies and literature reviews have attempted to determine correlates of protection for filovirus vaccines and to define the ability of humoral or cellular immunity to ameliorate disease [8–12]. Not surprisingly, it appears that both the humoral and cellular arms of the immune response can contribute to protection.

We have recently developed (a) replication-competent, (b) replication-deficient, and (c) chemically inactivated rabies virus (RABV) vaccines expressing EBOV (Zaire) glycoprotein (GP) [13]. The recombinant RABV vaccine vector (RVA) is derived from the SAD B19 strain which is used for wildlife vaccination in Europe and has previously been used as a safe and efficacious platform to generate vaccine candidates against several pathogens [14–18]. Two live vaccine candidates, RV-GP and RVΔG-GP, which has a deletion removing the entire RABV glycoprotein (G) gene, were found to be avirulent upon peripheral administration in mice. Based on the efficient incorporation of GP into the virion, an inactivated vaccine, INAC-RV-GP, was also produced by treatment with beta-propiolactone, the standard method utilized for the current human RABV vaccine. Each bivalent vaccine candidate induced strong humoral immunity to RABV G and EBOV GP, and conferred protection from both lethal RABV and mouse-adapted EBOV challenge in mice [13]. Our primary focus is the development of an inactivated vaccine for use in humans based on the potential for superior safety and the history of the successful existing RABV vaccine that is widely used in humans, but we are also pursuing the live attenuated vaccine candidates for use in nonhuman primate populations in Africa at risk for lethal EBOV infection [19, 20].

Here, we expand our investigation of the immune response to the RABV vaccine candidates expressing EBOV GP. Three critical elements of an effective vaccine platform for the filoviruses were assessed: (a) the ability to induce EBOV-specific T-cell immunity, (b) coformulation of vaccine candidates to induce multivalent antibody responses, and (c) induction of GP-specific immunity in the presence of pre-existing vector immunity to the RABV vaccine.

2. Methods

2.1 Viruses

The recovery and propagation of the vaccine candidates used in this study have been described previously [13, 18]. The SADB19-derived BNSP333 virus serves as the parent rabies vaccine vector RVA (Figure 1). RV-GP expresses the EBOV Mayinga GP ectodomain and transmembrane domain fused to the RABV G cytoplasmic domain. Inactivated RV-GP (INAC-RV-GP) was generated by treatment of sucrose purified virus stocks with a 1:2,000 dilution of beta-propiolactone (BPL) overnight at 4°C followed by 30 min at 37°C. RVΔG-GP expresses intact EBOV Mayinga GP and contains a deletion in the RABV G gene requiring propagation on complementary cells which express RABV G. BPL inactivated INAC-RV-HC50 expresses a chimeric protein composed of the heavy chain carboxyterminal half (HC50) of botulinum neurotoxin A fused with 30 amino acids of

RABV G ectodomain (ED), transmembrane domain (TM) and cytoplasmic domain (CD) [18]. A recombinant vaccinia virus expressing EBOV Mayinga GP was constructed using published methods [21].

2.2 Immunization of mice

All mouse experiments were approved by the NIAID Division of Intramural Research Animal Care and Use Committee. Injections of 0.1 ml live or inactivated virus were administered via the intramuscular (i.m.) route, 0.05ml in each hind leg. Live vaccines were delivered 1×10^5 FFU, and 10 μ g of the killed vaccines, which are equivalent to approximately 10^9 FFU, were delivered on day 0 or on days 0 and 14. Groups of 10 BALB/c mice (Jackson Laboratories) were immunized with either vehicle, RVA (parent virus), RV-GP, RVΔG-GP, INAC-RV-GP or INAC-RV-GP with an additional dose at day 14. For analysis of primary T cell response, four mice per group were sacrificed at day 7 post-immunization, and splenocytes were assayed for each individual mouse by ELISPOT (Figure 2A). For analysis of the memory recall response, six mice per group were challenged 28 days after immunization by intraperitoneal (i.p.) inoculation with 1×10^7 PFU vaccinia virus expressing EBOV GP. Spleens were removed five days later and assayed for each individual mouse by ELISPOT (Figure 2B).

For analysis of humoral immunity, groups of five Balb/c mice were immunized at day 0 (1x) or day 0 and 14 (2x) with 10 μ g of single inactivated vaccines or 20 μ g of co-formulated INAC-RV-GP + INAC-RV-HC50 (10 μ g each virus). For analysis of the ability to induce EBOV GP-specific humoral immunity in the presence of RABV immunity, groups of five Balb/c mice were immunized with 10 μ g INAC-RV-HC50 followed by immunization with 10 μ g INAC-RV-GP 28 days later. In these experiments, serum was collected four to six weeks post-immunization for individual analysis, although volume restraints required sera to be pooled for the HC50 group.

2.3 Interferon- γ ELISPOT

Single cell suspensions of splenocytes were prepared as previously described [22]. The mouse IFN γ ELISPOT kit (R&D Systems) was used for this assay. Plates were blocked with complete medium (Iscoves MDM supplemented with 10% FBS and 50uM beta-mercaptoethanol) for 2 hours at room temperature. Blocking media was removed and antigens diluted in fresh complete media were added to respective wells: an EBOV GP peptide pool or Influenza NP (a. a. 147–155; TYQRTRALV) at 10ug/ml. The EBOV GP peptide pool consisting of 167 15mers overlapping by 11 amino acids was acquired from JPT Peptide Technologies. Unstimulated wells contained complete media only. One hundred thousand cells were added to each well, and plates were incubated for 24 hours in a humidified incubator at 37C, 5% CO₂. Plates were then washed and processed according to manufacturer's instructions, and spots were enumerated using an ImmunoSpot reader and ImmunoSpot software (Cellular Technology Ltd.).

2.4 ELISA

Humoral immunity was assessed by ELISA against RABV G, EBOV GP, and botulinum neurotoxin HC50. Briefly, Maxisorp 96 well ELISA plates (Nunc) were coated with respective antigen overnight at 4°C as previously described [13, 18]. Coating buffer was removed, and plates were washed 4x with PBS + 0.1% Tween. Sera were diluted in three- or four-fold increments, and plates were incubated overnight at 4°C. Washes were repeated, and secondary HRP-conjugated antibodies were added respectively. After 1h at RT, washes were repeated, and substrate was added to each well. Plates were incubated for 2–15 min at room temp. Stop solution was added and OD490 was determined using a plate reader.

2.5 Statistical Analyses

Data were analyzed by Prism software (Graphpad). For ELISPOT results, groups were compared via one-way ANOVA and with Dunnett's Multiple Comparison test using RVA as the control. Unpaired two-tailed t-tests were used for ELISA data analysis with Welch's correction if variances were unequal.

3. Results

3.1 Cellular immune response to RVAs expressing EBOV GP

We have previously demonstrated that both live and inactivated RABV vaccines expressing GP are potent inducers of humoral immunity against both RABV G and ZEBOV GP [13]. However, there is evidence from previous vaccine strategies that T-cell mediated immunity may be important for the induction of protective immunity against the filoviruses [11, 12]. Therefore, we have attempted to determine if our live and killed vaccine candidates induce primary and memory GP-specific T-cells using a murine interferon- γ ELISPOT assay with a GP peptide pool or an irrelevant influenza peptide as stimulation. For the primary response at day 7 post-immunization (Figure 2A), each live and inactivated vaccine candidate was found to induce GP-specific, interferon- γ -expressing splenocytes above levels observed in the vehicle or RVA control groups. When compared to RVA, immunization with live RV-GP resulted in a significantly higher level of interferon- γ -expressing splenocytes ($p < 0.001$; mean of 340 spots per million cells (spmc)), while RV Δ G-GP and one or two doses of INAC-RV-GP resulted in a mean number of 35–50 spmc.

A critical measure of the cellular immune response is the ability to recall functionally active T cells upon viral challenge. Therefore, we analyzed the memory recall T-cell response in immunized mice after challenge i.p. with 1×10^7 PFU vaccinia virus expressing EBOV GP, which serves as BSL-2 surrogate challenge virus, at four weeks post-immunization. Immunization with RV-GP, RV Δ G-GP, or INAC-RV-GP 1x or 2x induced a recall response as detected by the higher level of GP-specific, interferon- γ -expressing splenocytes when compared to the vehicle or RVA control groups. As observed in the primary response, RV-GP induced a significantly higher level of memory T cells than RVA (mean of 535 spmc, $p < 0.001$). The replication-deficient virus, RV Δ G-GP, and the inactivated vaccine, INAC-RV-GP, also induced elevated T cell responses (mean of 270 and 285 spmc, respectively). Additionally, two doses of INAC-RV-GP induced a recall T cell response at levels comparable to the live vaccines, which was significantly higher than the RVA response (mean of 486 spmc, $p < 0.01$).

3.2 Inactivated vaccines induce multivalent humoral immunity

We have previously demonstrated that RABV vaccines expressing GP effectively induce bivalent RABV G-specific and EBOV GP-specific antibody responses [13]. However, an effective filovirus vaccine will likely need to confer immunity to several viral species [23]. We next sought to determine if co-administration with an additional RABV vectored vaccine would result in induction of a multivalent antibody response against three vaccine antigens. As a proof of principle experiment, we utilized a previously reported inactivated RABV vectored vaccine which expresses a fragment of the botulinum neurotoxin A termed HC50 to co-administer with INAC-RV-GP to determine if multivalent antibody responses against RABV G, botulinum HC50, and EBOV GP could be induced.

Groups of five mice were immunized i.m. once (day 0) or twice (day 0 and 14) with 10 μ g of INAC-RV-GP or INAC-RV-HC50 or 20 μ g for the combined administration (10 μ g each virus). As indicated in Figure 3A, each vaccination approach induced strong antibody responses against RABV G as expected since RABV G was present in each immunogen.

Either a single dose or two doses of INAC-RV-HC50 induced botulinum HC50-specific antibodies, and interestingly, combined administration with INAC-RV-GP resulted in a slightly stronger HC50-specific response (Figure 3B). Finally, analysis of the GP-specific antibody response indicated that single or boosted immunization with INAC-RV-GP induced strong immunity as expected (Figure 3C). Importantly, co-administration of INAC-RV-GP and INAC-RV-HC50 induced antibody levels that were nearly identical to INAC-RV-GP immunization. These results indicate that a potent multivalent response can be induced by this inactivated vaccination platform. Co-immunization with three antigens, RABV G, HC50, and ZEBOV GP resulted in no decrease in antibody response against each individual immunogen.

3.3 Induction of GP-specific antibodies in the presence of RABV immunity

There is a possibility that some members of the target population for an Ebola vaccine such as lab workers or first responders may be previously vaccinated with the currently approved RABV vaccine and thus have pre-existing immunity to RABV. This pre-existing immunity might interfere with induction of the EBOV GP-specific antibodies upon immunization with INAC-RV-GP. Therefore, we sought to determine in the mouse model if prior vaccination with a RABV vaccine would inhibit the induction of GP-specific antibodies (Figure 4). Groups of five mice were immunized once on day 0 with vehicle, 10 μ g INAC-RV-HC50 or INAC-RV-GP. A fourth group was immunized with 10 μ g inactivated INAC-RV-HC50 on day 0 followed by 10 μ g inactivated INAC-RV-GP on day 28. Four weeks after immunization, serum from each group was assayed by ELISA against (A) RABV G, (B) HC50, and (C) EBOV GP. As expected, each vaccination approach induced strong antibody responses against RABV G (Figure 4A) and vaccination with INAC-RV-HC50 or INAC-RV-HC50 followed by INAC-RV-GP induced potent HC50-specific antibodies (Figure 4B). Interestingly, vaccination with INAC-RV-HC50 followed by INAC-RV-GP induced similar levels of GP-specific antibodies to vaccination with INAC-GP alone (Figure 4C). These results indicate that immunization with INAC-RV-GP can induce GP-specific antibodies in the presence of pre-existing RABV immunity. The presence of a potent RABV G-specific antibody response at day 28 prior to immunization with INAC-RV-GP was confirmed (data not shown).

4. Discussion

Several vaccination strategies have been demonstrated to confer protection from Ebola hemorrhagic fever in macaques, including DNA vaccines, virus-like particles, or recombinant viruses expressing GP including adenovirus, vesicular stomatitis virus, or paramyxoviruses [2, 4–8, 24–28]. As expected, each vaccine candidate comprises advantages and disadvantages with regard to safety concerns, pre-existing vector immunity, manufacturing, dosage/schedule issues, or the ability to induce various components of the immune response. The experiments described here were designed to build upon our initial findings that live and inactivated RABV vaccines expressing GP induced strong humoral immunity and conferred protection from both RABV and EBOV in mice [13]. The studies sought to support more thorough future investigation of immunity and protective efficacy in macaques, which are believed to serve as the best animal model for study of filovirus hemorrhagic fever based on the similar disease presentation as observed for humans.

The contribution of T-cell mediated immunity to protection from EBOV challenge in mice and macaques has been recently reviewed and appears to vary among the vaccine candidates [11, 12]. The cellular immune response has been suggested to contribute to protection in mice for virus-like particle vaccines, but not for vesicular stomatitis virus based vaccines [29, 30]. Recently, protection in macaques mediated by adenovirus vectored GP was attributed to CD8⁺ T-cells by depletion prior to challenge [10]. However, some protective

vaccines in macaques are not believed to induce strong cellular immunity [12]. Here, investigation of the T-cell response to the RVA-vectored GP vaccines was pursued for comparison to other candidates. Both live and killed vaccines induced primary T-cell mediated responses as measured by interferon- γ ELISPOT with the response to RV-GP being the most robust. As a means to study the memory recall response in the absence of a BSL-4 facility, we used a vaccinia virus expressing EBOV GP as a surrogate challenge virus. Again, each vaccine candidate induced high levels of recalled GP-specific T-cells upon challenge, and a two doses regime of INAC-RV-GP was found to induce T-cells on par with RV-GP. As inactivated vaccines are commonly believed to be weak inducers of T-cell immunity, these data were very encouraging; particularly, since we are focusing on INAC-RV-GP for human vaccine development. It is important to note that INAC-RV-GP is inactivated by the same method as the RABV vaccine currently used for humans and requires no adjuvant. These results indicate that both live and killed vaccines induce T cell responses indicating that each of our vaccination strategies induces a potent humoral and cell mediated immune response.

We next sought to further define the humoral immune response to our lead candidate for human use, INAC-RV-GP, by assaying two critical parameters: the ability to induce multivalent immunity and immunity in the presence of pre-existing RABV vaccine vector immunity. For epidemiological and commercial considerations, an effective filovirus vaccine will likely require induction of multivalent immunity to Ebola virus (Zaire), Sudan Ebola virus, and Marburg virus. As cross-reactivity amongst the filoviruses is limited and not well-defined, vaccination with multiple immunogens is likely required and has been achieved with the DNA/adenovirus platform and the vesicular stomatitis virus platform [5, 31]. Here, as a proof-of-principle experiment, we demonstrated that co-administration of INAC-RV-GP with INAC-RV-HC50, an inactivated RABV vaccine which expresses a fragment of the botulinum neurotoxin, induced humoral immunity to RABV G, botulinum HC50, and EBOV GP that was comparable to single administration. Thus, the inactivated RABV vaccine platform appears to be well-suited for induction of multivalent immunity, and additional RABV vaccines expressing various filovirus GPs are being pursued.

Finally, by vaccinating RABV-immune mice with INAC-RV-GP, we demonstrated that pre-existing vector immunity to RABV did not prevent induction of GP-specific antibodies. The ability to effectively immunize mice in the presence of RABV G-specific antibodies suggests that our vaccination strategy may be effective in previously RABV-vaccinated humans and that boosting with various RABV vectored vaccines may be successful. This finding is important as many laboratory workers, first responders, or soldiers who might receive EBOV vaccination may be previously immunized with RABV vaccine. Although pre-existing immunity to VSV vectored vaccines would presumably be low and not an issue, pre-existing immunity in the general population to adenovirus and paramyxovirus vectored vaccines has been raised as a potential concern [2].

Taken together, these results further support the strong potential of using the RABV vaccine platform as means to develop inactivated filovirus vaccines for use in humans and live vaccines for use in nonhuman primates at risk for EBOV infection in Africa. Three critical parameters were demonstrated: induction of cell-mediated immunity, the ability to induce a multivalent humoral response, and the ability to immunize in the presence of vector immunity. Further definition of the immune response to these vaccine candidates will now shift to study in macaques. Should immunogenicity and efficacy studies in nonhuman primates result in positive outcomes, we believe that the RABV vaccine platform may be a superior strategy for filovirus vaccination based on consideration of safety, manufacturing, cost, and the ability to also confer protection from RABV which is still a major public health problem in Africa [32, 33].

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Highlights

- Both live and killed rabies/Ebola vaccines induce EBOV GP-specific CD8⁺ T-cells
- Co-formulation of inactivated vaccines induces multivalent humoral immunity
- Humoral immunity can be induced in the presence of pre-existing immunity to RABV

Figure 1.

Vaccine constructs. Negative-sense RNA genomes are illustrated for the parental RABV vaccine, RVA, and for its derivatives expressing Zaire Ebola virus strain Mayinga GP, RV-GP; RV-ΔG-GP, from which RABV G has been removed; and INAC-RV-GP, which has been killed via beta-propiolactone (BPL) inactivation. BPL inactivated INAC-RV-HC50 expresses a chimeric protein composed of the heavy chain carboxyterminal half (HC50) of botulinum neurotoxin A fused with 30 amino acids of RABV G ectodomain (ED), transmembrane domain (TM) and cytoplasmic domain (CD) [18]. The 333 mutation is an Arg→Glu change at amino acid 333 of RABV G, which has been shown to greatly attenuate neurovirulence of RABV vaccine vectors in adult mice [16].

Figure 2.

Live and killed RABV vaccines expressing EBOV GP induce (A) primary and (B) memory recall T-cell immunity. Balb/c mice were immunized i.m. with 5×10^5 FFU of the live viruses or 10 μ g of INAC-RV-GP (1x on day 0 or 2x on day 0 and day 14). (A) Primary T cell mediated immunity was analyzed at day 7 post-immunization. Splenocyte suspensions were generated for each mouse individually and used in a murine interferon- γ ELISPOT (R and D Systems) using an EBOV GP peptide pool or unrelated Flu control peptide as stimulation. (B) For analysis of memory T cell response, six mice per group were challenged i.p. with 1×10^7 PFU of vaccinia virus expressing GP at four weeks post-immunization. Five days later, spleens were removed and ELISPOT was performed as described above for the primary response. Results are mean spots per million cells, and error bars are standard error of the mean. ***, $p < 0.001$; **, $p < 0.01$.

Figure 3.

Analysis of co-administration of two inactivated vaccines: INAC-RV-GP and INAC-RV-HC50. Groups of five mice were immunized i.m. once (day 0) or twice (day 0 and 14) with the indicated viruses. Groups receiving 2 immunizations are labeled x2. Single virus doses were administered at 10 μ g, while the combined inactivated virus groups, labeled GP + HC50, were administered at 20 μ g (10 μ g of each virus). On day 42, all mice were bled and serum was assayed by ELISA against (A) RABV G, (B) botulinum HC50, and (C) EBOV GP. Results are graphed as mean absorbance values of the individual sera samples $\pm$ standard error, except for anti-HC50 (B) where sera were pooled due to volume constraints. Unpaired t-tests showed significant differences between respective single and double dose vaccinations as shown. **, $p < 0.01$; *, $p < 0.05$.

Figure 4.

Induction of GP-specific antibody response in the presence of pre-existing RABV immunity. Groups of five mice were immunized once on day 0 with vehicle, 10 μ g INAC-RV-HC50, or 10 μ g INAC-RV-GP. A fourth group was immunized with 10 μ g INAC-RV-HC50 on day 0 followed by 10 μ g INAC-RV-GP on day 28 (labeled HC50, then GP). Four weeks later (day 56), serum was assayed for each mouse individually by ELISA against (A) RABV G, (B) BoNT HC50, and (C) EBOV GP. Results are graphed as mean absorbance values of the individual sera samples $\pm$ standard error. For the anti-RABV G ELISA data (4A), unpaired t-tests showed significant differences for each sera dilution between the “HC50, then GP” group and either of the single dose groups. The higher of the two p-values is shown. ****, $p < 0.0001$; ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.