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Rabies virus glycoprotein as a carrier for anthrax protective antigen

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Abstract

Live viral vectors expressing foreign antigens have shown great promise as vaccines against viral diseases. However, safety concerns remain a major problem regarding the use of even highly attenuated viral vectors. Using the rabies virus (RV) envelope protein as a carrier molecule, we show here that inactivated RV particles can be utilized to present *Bacillus anthracis* protective antigen (PA) domain-4 in the viral membrane. In addition to the RV glycoprotein (G) transmembrane and cytoplasmic domains, a portion of the RV G ectodomain was required to express the chimeric RV G anthrax PA on the cell surface. The novel antigen was also efficiently incorporated into RV virions. Mice immunized with the inactivated recombinant RV virions exhibited seroconversion against both RV G and anthrax PA, and a second inoculation greatly increased these responses. These data demonstrate that a viral envelope protein can carry a bacterial protein and that a viral carrier can display whole polypeptides compared to the limited epitope presentation of previous viral systems.

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Introduction

Until recently infection with *Bacillus anthracis*, the causative agent of anthrax had posed only a low risk, with minimal natural occurrence in humans. However, the development of *B. anthracis* as a bioterrorism tool with the death of five people and the infection of at least seventeen others in the U.S. (Jernigan et al., 2002) in 2001, has underscored the danger of an anthrax attack. Existing

prophylactic and therapeutic strategies for combating anthrax are insufficient.

The most lethal form of the disease, inhalation anthrax, presents with general, flu-like symptoms, so that early diagnosis is difficult. Prompt recognition of symptoms is required for effective therapeutic treatment using antibiotics or passive immunization following unknown exposure (Sternbach, 2003). While passive immunization is effective (Sawada-Hirai et al., 2004), the availability of immune sera is highly limited and no approved therapeutic antibodies against anthrax are currently available. Several antibiotics are effective against *B. anthracis*, but, antimicrobial resistance engineered into future bioterrorism-grade anthrax can be envisioned. Moreover, antibiotics can eliminate the bacteria but not the toxins that may already have been released. Thus, vaccination is a highly practical alternative to post-exposure treatment and eliminates the risk of infection.

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Anthrax disease is caused by the toxins. The anthrax toxins act as a tripartite system in which transport of the active components, lethal and edema factors into the cell, is facilitated by the binding component, protective antigen (PA). PA binds to receptors on the cell surface and is cleaved by cellular furin (Molloy et al., 1992) into a small subunit and the larger 63-kDa constituent, PA63. Seven receptor-bound PA63 units oligomerize to form a complex that binds with lethal factor and edema factor and that passes into the cytoplasm. Two receptors, ATR/TEM8 (Bradley et al., 2001) and CMG2 (Scobie et al., 2003), bind PA and mediate toxicity. PA is comprised of four distinct domains, with receptor interactions occurring at sites within PA domain-4 (D4) and -2. The structure of the receptor binding ridge is highly conserved, suggesting that ATR/TEM8 and CMG2 interact with PA in the same manner (Santelli et al., 2004). Passive and active immunization indicates that protection is mediated by humoral immune responses (Enserink, 2002; Pitt et al., 2001; Welkos et al., 2001) and binding of PA to the cell surface receptors can be prevented by anti-PA antibodies. Of note, antibodies elicited by PA immunization can confer protection against *B. anthracis* challenge (Beedham et al., 2001; Ivins et al., 1992, 1998; Kobiler et al., 2002; Little et al., 1997), and antibodies against epitopes within PA D4 also show protective capacity (Flick-Smith et al., 2002b; Kasuya et al., 2005; Maynard et al., 2002).

An FDA-licensed vaccine against anthrax exists consisting of a cell culture filtrate adsorbed onto aluminum hydroxide (Bioport, 2002). However, several aspects of this product point to need for development of a novel vaccine: (i) it is indicated only for use in at-risk individuals; (ii) the material is difficult to characterize (Turnbull, 2000); (iii) it requires six immunizations within the first 1.5 years with annual boosters thereafter (Bioport, 2002); and (iv) reactogenicity is reported in up to 35% of patients (Bioport, 2002).

Additional vaccine strategies, most of which focus on PA, are being pursued. These include DNA vaccines encoding PA (Ferrari et al., 2004), as well as approaches using various microorganism vectors to express PA, such as influenza (Li et al., 2005), vaccinia virus (Iacono-Connors et al., 1991), adenovirus (Tan et al., 2003), Venezuelan equine encephalitis virus-based replicons (Lee et al., 2003) and a *Salmonella enterica* serovar typhimurium live vaccine encoding domain four of PA (Galen et al., 2004). The best-developed vaccine candidate is a recombinant PA (rPA) adsorbed to aluminum hydroxide (Keyserling et al., 2004). Efforts to enhance the immunogenicity of rPA include immunogenic formulations such as copolymers (Coeshott et al., 2004) or microsphere encapsulation (Flick-Smith et al., 2002a) or addition of adjuvants (Berthold et al., 2005; Ivins et al., 1992, 1998; McBride et al., 1998).

Rabies virus is a promising vaccine vector able to induce humoral and cellular immune responses efficiently to foreign antigens (McGettigan et al., 2001a, 2001b; Schnell et al., 2000). Because of low seroprevalence in the human population, RV is an excellent viral vector candidate. Methods for engineering the virus are well established, up to two foreign genes totaling 6.5 kb have been incorporated thus far, and foreign sequences

are stably maintained. RV grows to high titers in cell lines approved for human vaccine production and manufacture is economical. Our current RV vaccine vector is based on the vaccine strain SAD-B19 used since 1983 as a live oral immunogen to vaccinate wild life in Europe and Asia (Schneider et al., 1988). To further increase safety, several attenuating mutations have been introduced into these live viral vectors (McKenna et al., 2003).

Inactivated RV has been used to vaccinate humans in the U. S. since 1978 (Plotkin, 1980) and thus has a significant history of safety (Haupt, 1999). Potent and long-lasting neutralizing antibodies to the RV glycoprotein are generated by immunization with killed particles (Thraenhart et al., 1994). Strong humoral responses to heterologous glycoproteins such as HIV-1 envelope (McKenna et al., 2004; Schnell et al., 2000) and HCV E1/E2 (Siler et al., 2002) are elicited by killed RV particles incorporating the foreign proteins. The highly repetitive and rigid organization of viruses and virus-like particles is thought to maximally stimulate B cells (Fehr et al., 1997; Jegerlehner et al., 2002). CD4⁺ T helper epitopes provided by virus particles can also provide necessary signals for B cell activation (Hooper et al., 1994).

Here, we generated several RV G-PA chimeras in which either PA63 or PA D4 was fused to ectodomain (ED) truncation mutants of RV G. One of these chimeras, D4-E51, properly trafficked to the cell surface and was efficiently incorporated into virus particles. Mice immunized with killed SPBN-D4-E51 particles mounted high antibody titers against *B. anthracis* PA. The response was dose-dependent and was skewed toward Th2-associated antibodies. Lymphocyte proliferation was also observed, in response to viral antigens and anthrax PA. These results suggest that inactivated rabies virus can serve as an immunostimulatory carrier for immunization against pathogens such as anthrax.

Results

Rabies virus expressing anthrax PA63

Anthrax PA is an excellent target for an anthrax vaccine because particular anti-PA antibodies can prevent entry of the toxin into cells. Therefore, anthrax PA63, optimized for human codon usage to ensure high expression levels, was cloned into the RV vaccine vector pSPBN resulting in pSPBN-PA63 (Fig. 1). Recombinant RV was recovered by standard methods and expression of PA63 was confirmed by immunostaining with anti-PA monoclonal antibody (Fig. 2, panel B'). To analyze the immunogenicity of the recombinant RV SPBN-PA63, five BALB/c mice were inoculated i.m. with 10⁶ ffu and sera from immunized mice were analyzed by a PA-specific ELISA 14 days after immunization. However, no PA-specific antibodies were detected in any of the immunized animals (data not shown). To determine whether surface expression of anthrax PA might increase its immunogenicity in the vaccinated host, we used the RV G ER translocation SS in addition to the RV G TM and CD (Fig. 1). The gene encoding the chimeric RV G-anthrax PA was introduced into

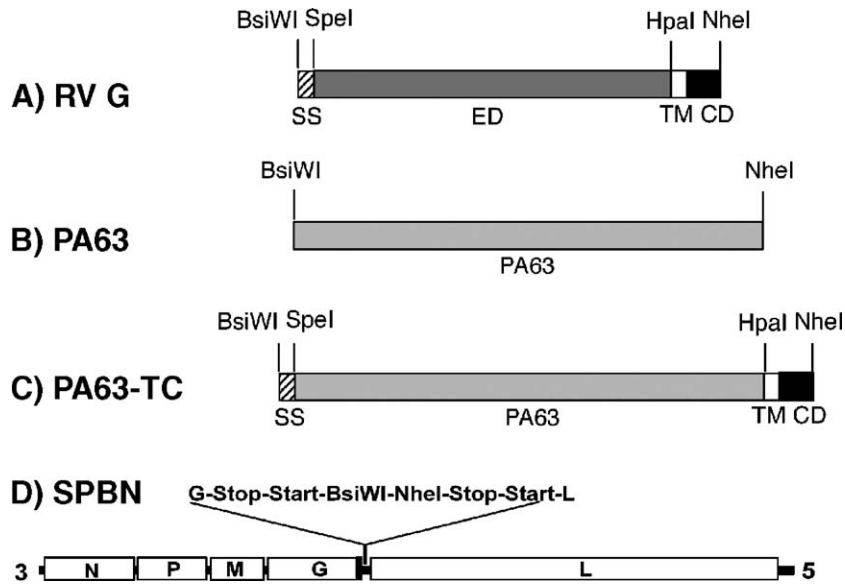


Fig. 1. Construction of recombinant RVs expressing anthrax PA63 or chimeric RV G/anthrax PA63 fusion protein. *SpeI* and *HpaI* restriction sites flanking the RV G ED between RV G SS and TM were introduced into the RV G gene (A) and were the target to replace the RV G ED sequence with PA63, resulting in PA63-TC (C). *BsiWI* and *NheI* sites (B) were used to insert PA63 into the SPBN vector (D).

the RV vector pSPBN, resulting in pSPBN-PA63-TC. BSR cells were infected with SPBN (Fig. 2, panels A, A', A''), SPBN-PA63 (Fig. 2, panels B, B', B'') or SPBN-PA63-TC

(Fig. 2, panels C, C', C'') at an MOI of 0.01 and fixed 48 h later with paraformaldehyde. Infected cells were analyzed directly by immunofluorescence microscopy with a monoclonal

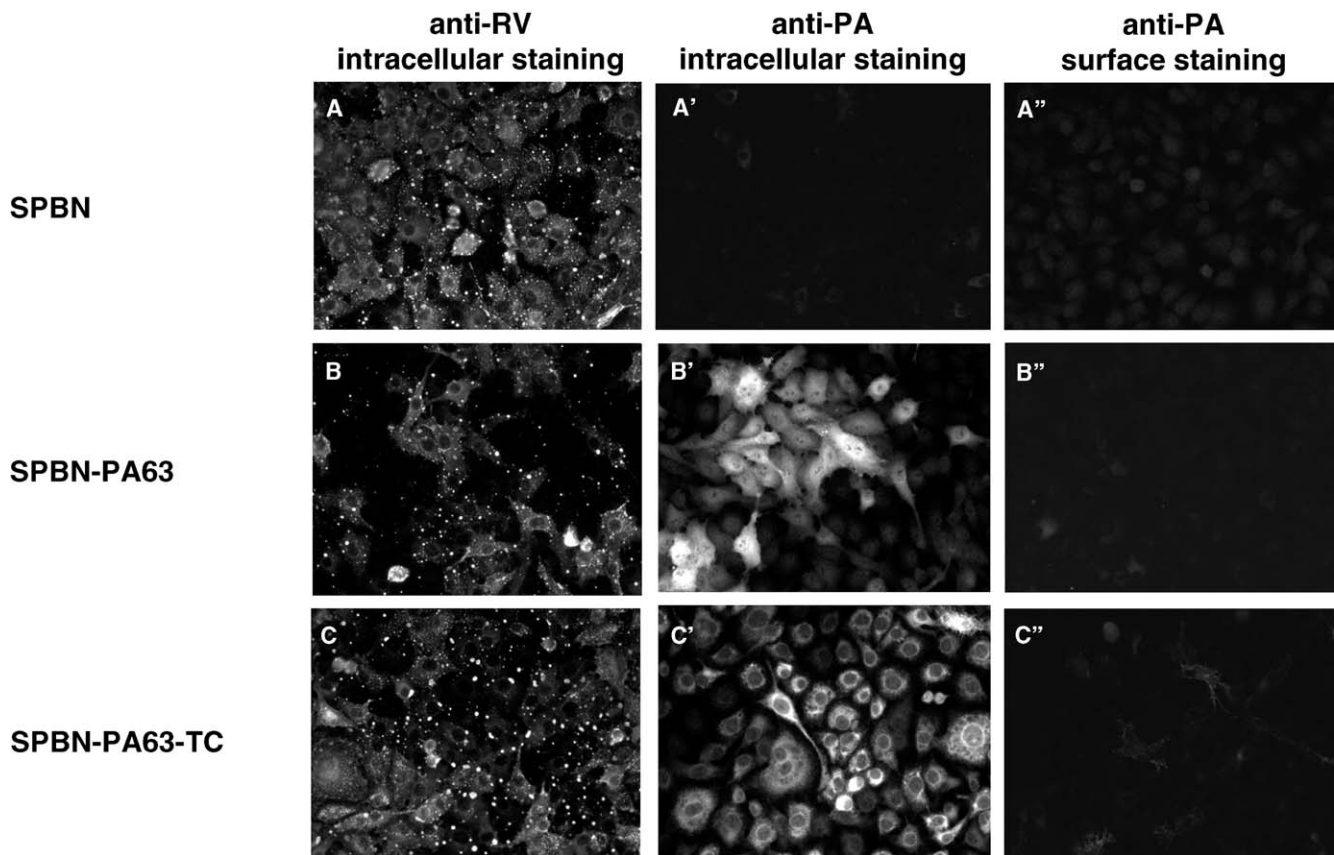


Fig. 2. Immunofluorescence for protein expression and surface staining. BSR cells were infected with SPBN (Panels A, A', A''), SPBN-PA63 (Panels B, B', B'') or SPBN-PA63-TC (C, C', C'') at an MOI of 0.01 and fixed 48 h later with paraformaldehyde. Infected cells were analyzed directly by immunofluorescence microscopy with a monoclonal antibody directed against PA (Panels A'', B'', C'') or permeabilized with Triton X-100 for internal staining with an antibody against anthrax PA (Panels A', B', C') or RV N protein (Panels A, B, C).

antibody directed against PA (Fig. 2 panels A'', B'', C'') or permeabilized with Triton X-100 for internal staining with an antibody against anthrax PA (Fig. 2 panels A', B', C') or RV N protein (Fig. 2 panels A, B, C). All cells were infected with RV as indicated by a bright signal with an RV N-specific antibody. Moreover, anti-PA monoclonal antibody detected the internal expression of PA63 in both SPBN-PA63- or SPBN-PA63-TC-infected cells. However, no expression of PA63 on the cell surface was detected for either of the viruses expressing PA63 independent of the use of the RV G signal sequence.

Part of the RV G ED is required for surface expression of PA D4

To test the hypothesis that a lack of transport through the ER and Golgi apparatus accounted for the lack of surface expression, and that a portion of the RV G might support such transport, we utilized different portions of the RV G ED in addition to the TM and CD of RV G. We constructed five different plasmids containing increasing portions of the RV G ED in addition to anthrax PA. Based on previous research indicating that D4 of anthrax PA is the most potent antigen to induce protective antibodies, we used this 140-aa fragment comprising the carboxy-terminal region of PA. Use of this fragment allowed us to examine whether D4 flanked by the RV G SS, TM and CD fails to be transported to the cell surface as observed for PA63. The genes encoding RV G-anthrax PA D4 fusion proteins containing 0, 51, 127, 210, or 439 amino acids of the RV ED were PCR-amplified and introduced into an expression plasmid under the control of the T7 RNA polymerase promoter (Fig. 3). The resulting plasmids were designated pD4- Δ ED, pD4-E51, pD4-E127,

pD4-E210, and pD4-E439. To evaluate the cell surface expression of the recombinant RV G-D4 fusion proteins, BSR cells were infected with recombinant vaccinia virus expressing T7 RNA polymerase for one h, transfected with 5 μ g of each of the five plasmids and 24 h later fixed in paraformaldehyde, permeabilized, and immunostained with anti-PA monoclonal antibody. A second set of transfected cells was treated similarly but was not permeabilized. As shown in Fig. 4A, all proteins were successfully expressed (internal staining) in transfected cells, whereas significant surface expression was detected only for recombinant protein D4-E51 (surface staining). More quantitative analysis by FACS (Fig. 4B) confirmed that only D4-E51 was expressed at high levels on the cell surface, while surface expression for pD4-pD4-E127 and pD4-E439 was detectable but at lower levels than observed for pD4-E51. Western blotting analysis of transfected cell lysates using antibodies directed against anthrax PA or RV G CD (Fig. 4C) revealed chimeric RV G-D4 proteins of the expected sizes of 21 kDa, 29 kDa, 37 kDa, or 74 kDa for D4- Δ ED, D4-E51, D4-E127, and pD4-E439 with both the PA- and the RV G CD-specific antibodies. Interestingly, the RV G CD-directed antibody failed to detect the D4-E210 fusion protein suggesting cleavage or degradation of the carboxy-terminus of this protein. The antibody directed against the amino-terminal-located D4 of D4-E210 detected the protein but at a smaller than predicted size, further indicating degradation. All recombinant fusion proteins showed a diffuse migration pattern over a range of up to 20 kDa. Sequence analysis of PA indicates several potential N- and O-glycosylation sites within D4, which might contribute to the observed retarded migration.

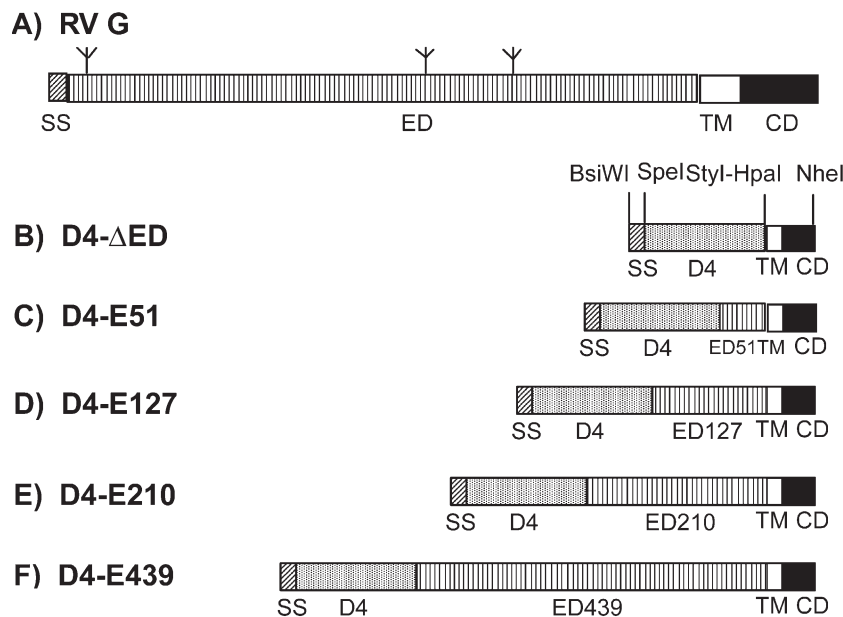


Fig. 3. Construction of plasmids containing D4 fused to various RV G ED fragments. Panel A shows RV G protein is shown in panel A. The three potential sites of N-linked glycosylation are indicated. To construct chimeric RV G-anthrax PA fusion proteins, the RV G ED was replaced with domain 4 of PA (D4- Δ ED, B). The membrane-proximal 51, 127 or 210 amino acids of the RV G ectodomain containing one, two or three potential N-linked glycosylation sites, respectively, were reintroduced into D4- Δ ED to generate D4-E51, D4-127 and D4-210, respectively (C, D, E). The full-length RV G ectodomain was inserted into D4- Δ ED resulting in D4-E439 (F).

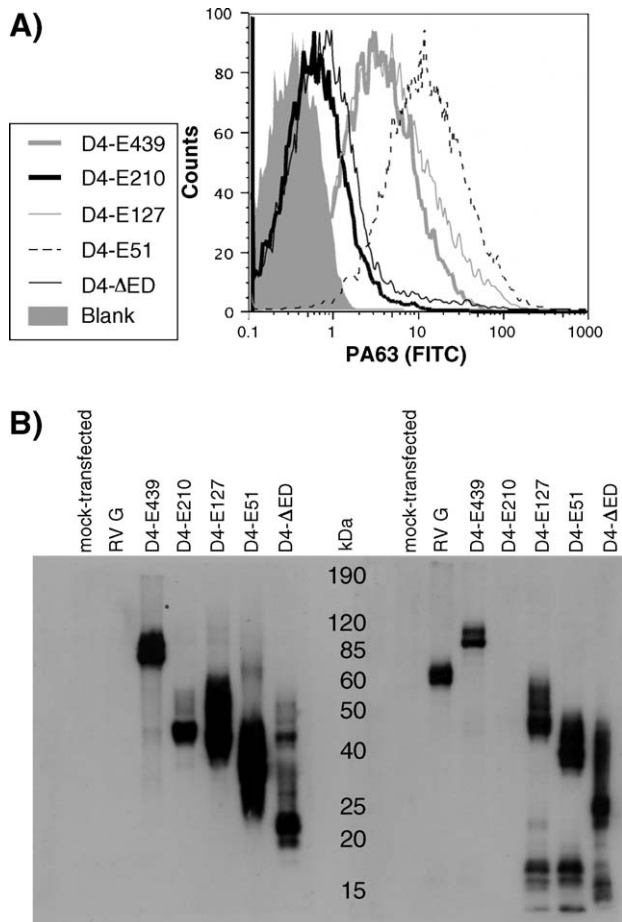


Fig. 4. Analysis of chimeric RV G/anthrax PA fusion proteins. BSR cells were infected with recombinant vaccinia virus expressing T7 TNA polymerase and transfected overnight with pD4- Δ ED, pD4-E51, pD4-E127, pD4-E210 or pD4-E439. To analyze surface expression of the fusion proteins, transfected cells were fixed in 3% paraformaldehyde and either permeabilized (internal staining) with 1% Triton X-100 or left untreated (surface staining). Cells were stained with mouse anti-PA monoclonal antibody followed by FITC-labeled donkey anti-mouse and examined by fluorescence microscopy (A). For FACS analysis of transfected cells was stained with mouse anti-PA followed by FITC-labeled donkey anti-mouse, fixed with formaldehyde and analyzed by flow cytometry (B). For Western blot analysis, lysates of transfected cells were prepared, separated by SDS-PAGE, and transferred to a nitrocellulose membrane. Blots were probed with either sera directed against the RV G CD (right panel) or anti-PA monoclonal antibody (left panel).

Chimeric RV G-D4-E51 protein is efficiently incorporated into RV virions

The results above indicated the highest level of surface expression for D4-E51, suggesting its suitability for incorporation into the RV envelope. Thus, the gene encoding D4-E51 was cloned into pSPBN resulting in pSPBN-D4-E51. Recombinant RV was recovered and analyzed for expression of the transgene in infected cells and composition of the recombinant virions. Western blotting analysis (Fig. 5) of lysates from SPBN- or SPBN-D4-E51-infected cells or sucrose-purified virions using anti-PA (Fig. 5 lanes 3, 4, 7, and 8) or anti-RV G CD (Fig. 5 lanes 1, 2, 5, and 6) revealed D4-E51 in cells infected with SPBN-D4-E51 or in purified SPBN-D4-E51 virions, whereas no signal was

observed for SPBN (Fig. 5, lanes 3 and 7). As expected, the RV G CD-specific antibody detected RV G for both viruses. An additional protein migrating at the expected size for the D4-E51 was detected in the case of SPBN-D4-E51-infected cells or SPBN-D4-E51 virions (Fig. 5, lanes 2 and 6).

Live and killed RV-based anthrax vaccines are immunogenic in mice

The successful incorporation of D4-E51 into virions enabled us to study the induction of anti-PA immune responses using both inactivated and live SPBN-D4-E51 in an animal model. Three groups of five female Swiss Webster mice were inoculated i.m. with 3×10^6 ffu SPBN-D4-E51, 50 μ g inactivated SPBN-D4-E51, 3×10^6 ffu SPBN or 50 μ g inactivated SPBN. Blood samples were obtained from all mice at 14 days post-immunization and sera were analyzed by ELISA on plates coated with rPA or RV G. As shown in Fig. 6B all immunized mice seroconverted against RV G. Moreover, all mice primed with inactivated or live SPBN-D4-E51 also mounted anthrax PA-specific antibodies, which were not detected in sera from mice immunized with SPBN. A second inoculation increased the RV G-specific titers about 3-fold, whereas the anthrax PA-specific ELISA titers were increased 10-fold, with no significant differences in antibody titers between mice immunized with live RV or inactivated RV virions (Fig. 6).

Several vaccine approaches that appear promising in mice are difficult to reproduce in larger animals or humans due to the large amount of antigen needed for immunization. Thus, we performed a dose-response analysis using antigen in amounts

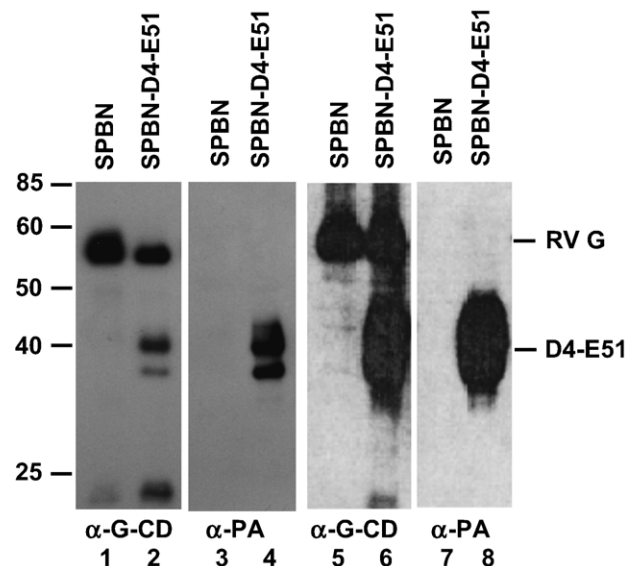


Fig. 5. Expression and incorporation of D4-E51 into RV particles. BSR cells were infected with SPBN or SPBN-D4-E51 at an MOI of 1 and after 48 h cell lysates were prepared, separated by SDS-PAGE, and transferred to a nitrocellulose membrane (lanes 1–4). To analyze incorporation of D4-E51 into RV virions, sucrose-purified SPBN or SPBN-D4-E51 particles were separated by SDS-PAGE and transferred to a nitrocellulose membrane (lanes 5–8). Blots were probed with either sera directed against the RV G CD (α -G-CD) or anti-PA monoclonal antibody (α -PA).

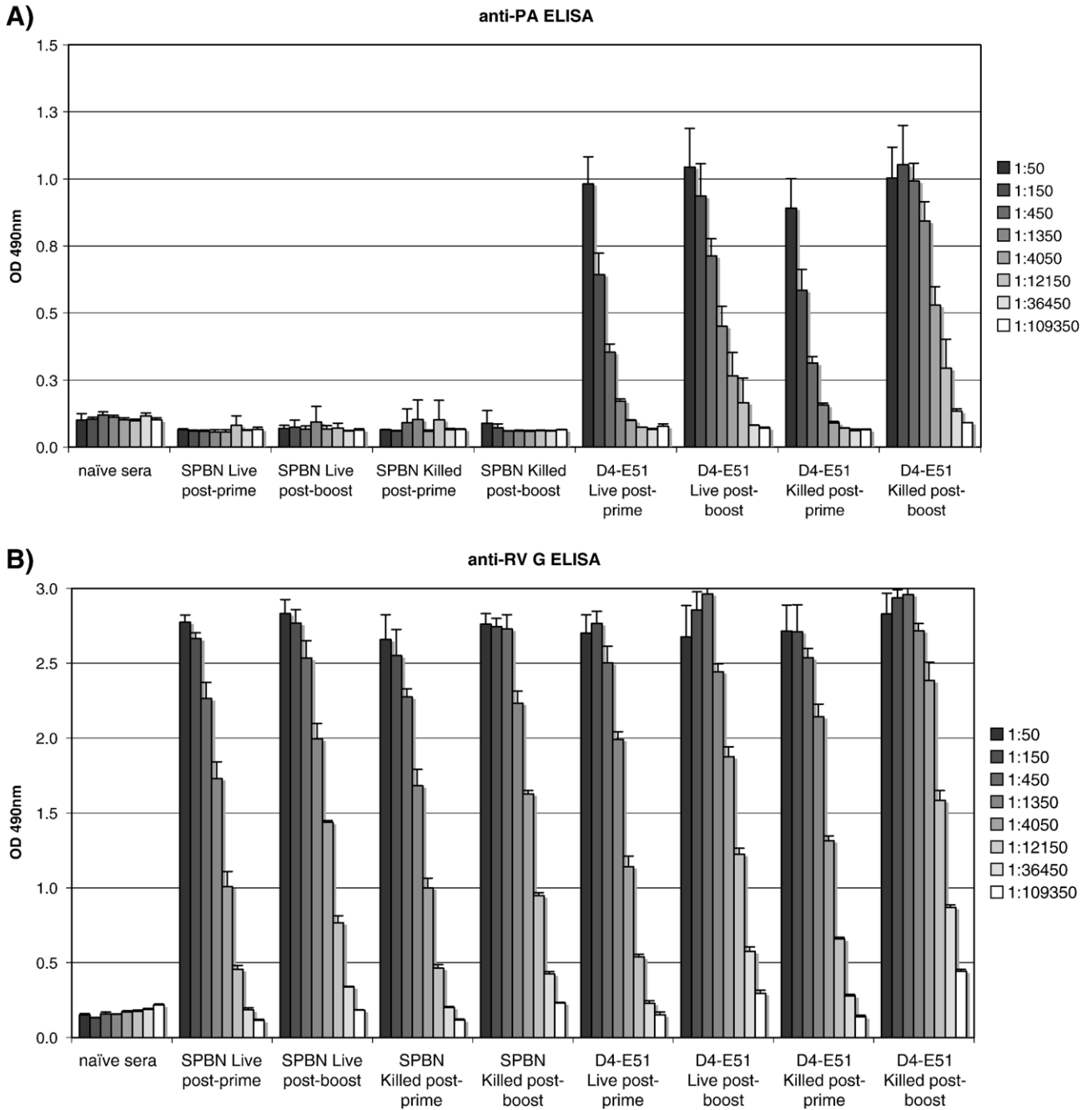


Fig. 6. Humoral immune response to live or inactivated SPBN-D4-E51. Groups of five mice were immunized i.m. with 50 µg of inactivated SPBN-D4-E51, 3 × 10⁶ ffu of live SPBN-D4-E51, 50 µg of inactivated SPBN, or 3 × 10⁶ ffu of live SPBN at 0 and 21 days. Mice were bled 2 weeks after each immunization and sera was analyzed by ELISA specific for anthrax PA (A) or RV G (B). Data represent the mean of triplicate dilutions prepared from pooled sera.

ranging from 50 µg to 5 ng. A mixture of 100 ng recombinant PA and 100 ng of RV G was included as a control. Groups of three mice were immunized with the respective antigen on days 0 and 21, bled 14 days post-immunization, and sera were analyzed by RV G- and anthrax PA-specific ELISAs (Table 1). All mice immunized with killed SPBN-D4-E51 in a range of 50 µg to 50 ng showed strong responses to RV G and anthrax PA. Moreover, immunization with as little as 5 ng resulted in antigen-specific seroconversion. In response to immunization with rPA and RV G, mice mounted titers similar to the 5 ng dose of SPBN-D4-E51. A second inoculation with the same vaccine

used for priming significantly increased the PA and RV G responses. In addition, antibodies levels in sera of mice immunized twice with recombinant PA and RV G were comparable to those in sera of mice immunized twice with 50 ng SPBN-D4-E51. A separate experiment (experiment B in Table 1) using the same antigens and schedule but with 5-fold increased amounts of recombinant anthrax PA and RV G showed similar results with the exception that the increased amount of RV G protein induced higher antigen-specific seroconversion. However, priming with 500 ng PA still resulted in low PA-specific titers after priming.

Table 1
Antibody titers of mice immunized with different amounts of inactivated SPBN-D4-E51

	Anti-PA response post-prime		Anti-PA response post-boost	
50 µg SPBN-D4-E51	A	13,575 (13,516–13,690)	A	>109,350
	B	1856 (1201–3730)	B	16,555 (9773–35,464)
5 µg SPBN-D4-E51	A	4768 (4616–4933)	A	>109,305
	B	1240 (497–3467)	B	24,622 (13,734–37,292)
500 ng SPBN-D4-E51	A	4801 (4678–5014)	A	39,154 (37,879–40,564)
	B	295 (129–1321)	B	4405 (1617–12,566)
50 ng SPBN-D4-E51	A	1439 (1365–1544)	A	14,397 (13,372–14,969)
	B	456 (44–1492)	B	655 (45–3905)
5 ng SPBN-D4-E51	A	130 (125–134)	A	1114 (941–1389)
	B	<50	B	156 (50–431)
100 ng rPA	A	115 (109–124)	A	13,645 (13,067–14,199)
500 ng rPA	B	451 (165–1183)	B	109,155 (84,899–142,221)
	Anti-RV G post-prime		Anti-RV G post-boost	
50 µg SPBN-D4-E51	A	>109,350 *	A	>109,350 *
	B	>109,350 *	B	>109,350 *
5 µg SPBN-D4-E51	A	>109,350 *	A	>109,350 *
	B	>109,350 *	B	>109,350 *
500 ng SPBN-D4-E51	A	14,831 (14,252–15,262)	A	>109,350 *
	B	24,122 (11,858–39,938)	B	>109,350 *
50 ng SPBN-D4-E51	A	13,698 (13,091–14,477)	A	12,908 (12,748–13,218)
	B	12,445 (11,809–12,938)	B	>109,350 *
5 ng SPBN-D4-E5	A	2154 (1527–4216)	A	145,056 (142,656–147,620)
	B	868 (133–11,809)	B	36,879 (9257–140,172)
100 ng RV G	A	153 (140–171)	A	86,546 (44,130–129,912)
500 ng RV G	B	23,273 (10,681–89,647)	B	>109,350 *

Geometric mean titer (range low–high).

Two independent experiments (A and B) were performed. Experiment A: groups of three mice were immunized i.m. with 50 µg, 5 µg, 500 ng, 50 ng or 5 ng of inactivated SPBN-D4-E51 or with 100 ng RV G + 100 ng rPA at 0 and 21 days. Mice were bled 2 weeks after each immunization. Sera for each group were pooled placed in triplicate ELISA wells and the geometric mean determined. Experiment B: mice were immunized and bled as in experiment A except that the protein group was immunized with 500 ng RV G + 500 ng rPA. Sera from individual mice were run in ELISA and the geometric mean determined.

* All three mice yielded titers >109,350.

Killed RV virions induce a predominant T helper type 2 cell-associated antibody response

To determine whether there were differences in IgG subclasses induced by our immunization approach, sera from the dose–response immunization regimen used in experiment B above were analyzed by a PA-specific ELISA for IgG1 and IgG2a. As shown in Fig. 7, immunization with the killed particles elicited predominantly IgG1, whereas immunization with “protein only” resulted in a high level of IgG2a compared to IgG1. Although Th2 cells are known to facilitate antibody production it remains whether such cells underlie the potent seroconversion after priming with inactivated virions.

Antigen-specific proliferative response after immunization with SPBN-D4-E51

To analyze the memory immune responses elicited by vaccination with this novel vaccine carrier, spleens of four 6- to 8-week-old Swiss Webster mice either left unimmunized (Fig. 8, open bars) or immunized i.m. with 50 µg of inactivated SPBN-D4-E51 (Fig. 8, shaded bars) on days 0 and 21 were harvested 4 weeks post-boost and Splenocytes were stimulated with RV G, RV RNP, inactivated virions (SPBN) or

recombinant PA (see Materials and methods). In three out of four immunized mice, the proliferative responses were greatly increased after restimulation with RV-specific antigens, whereas one mouse mounted no detectable response. Comparison of the two groups showed that such responses differed 11-fold for restimulation with inactivated RV particles ($P < 0.07$) and 15-fold for RV RNP ($P < 0.02$). The immunized group also responded to RV G ($P < 0.15$). Note the very substantial proliferative responses (11-fold, $P < 0.03$) detected after restimulation with recombinant anthrax PA, indicating that such responses can also occur in the absence of the RV antigens.

Discussion

This report describes the development of a novel approach to create a potential vaccine against anthrax using inactivated RV as a carrier. Virus particles present surface antigens in a dense and repetitive array that maximally stimulates B cell proliferation and antibody production (Zinkernagel, 2003). Additionally, the virus particle can provide CD4+ T helper epitopes that supply the required second signal for B cell activation (Baumgarth, 2000). To incorporate these properties into vaccine against anthrax, we generated an RV particle that displays the

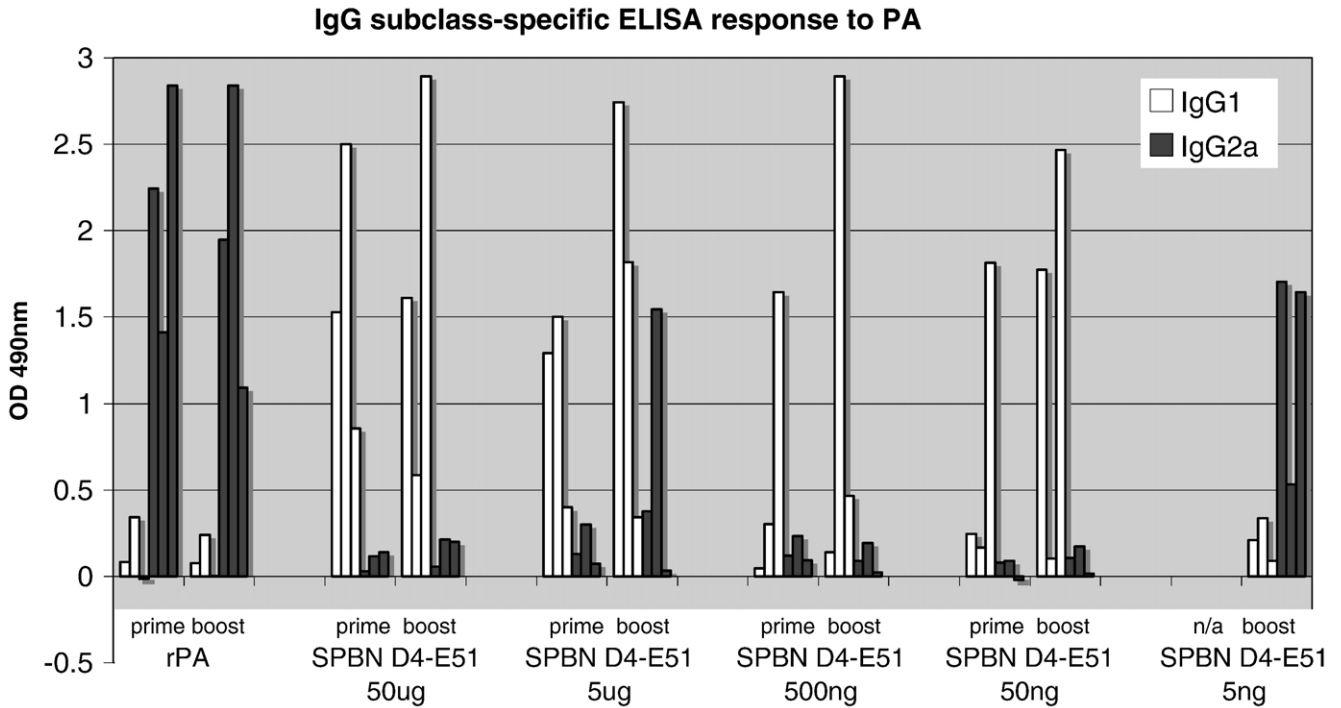


Fig. 7. IgG subclass induced by immunization with inactivated SPBN-D4-E51 or recombinant anthrax PA. Sera from the same mice as described in Table 1 were evaluated for anti-anthrax-PA-specific IgG1 and IgG2a responses by ELISA. Because no seroconversion was detected for 5 ng dose after priming for SPBN D4-E51, these sera were not analyzed (n/a).

antigenic component of *B. anthracis*, engineering the RV G such that PA is expressed on the surface of RV particles. While virus particles expressing chimeras of heterologous glycoproteins have been produced, the generation of viruses expressing viral glycoprotein-bacterial toxin fusion proteins is recent as exemplified by the insertion of PA D4 in the influenza virus

hemagglutinin (Li et al., 2005). RV G is a type I transmembrane protein and exists in virus particles as a homotrimer (Gaudin et al., 1992). The glycoproteins of RV and vesicular stomatitis virus (VSV), closely related rhabdoviruses, share 20% amino acid homology and their ectodomains are predicted to have similar three-dimensional structure (Whitt et al., 1991). Studies

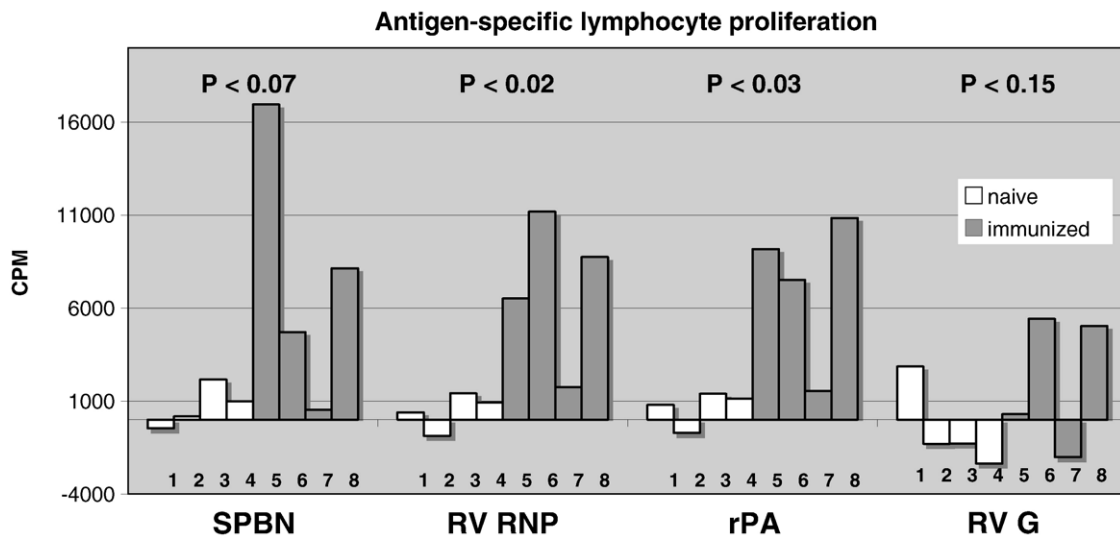


Fig. 8. Lymphoproliferative responses after immunization with inactivated SPBN-D4-E51. Groups of four 6- to 8-week-old Swiss Webster mice were either left unimmunized (open bars) or immunized i.m. with 50 µg of inactivated SPBN-D4-E51 (shaded bars) on days 0 and 21 and spleens were harvested 4 weeks post-boost. Splenocytes were stimulated with RV G, RV RNP or rPA for 3 days or with inactivated virions (SPBN) for 8 days. At the indicated time point, 100 µl of cells was removed and pulsed with 1 µCi of [3H]-thymidine. The average of three replicate wells is indicated in counts per minute (CPM) over background (medium only). Numbers below each bar reflect individual mice. Statistical significance was determined by Student's *t* test of the means of each group.

of VSV demonstrate the requirement for trimer formation in the endoplasmic reticulum for transport of VSV G to the cell surface (Doms et al., 1987, 1988). Consistent with these observations, our initial construct, PA-TC, as well as the D4- Δ ED lacking any of the RV G ectodomain, were not transported to the cell surface. Additionally, it has been shown that glycosylation of at least one site on the RV G ectodomain is required for trimer formation and for cell surface expression of wild-type RV G (Shakin-Eshleman et al., 1992). For VSV G, only the 12 membrane proximal amino acids of the ED are required for trimer formation, cell surface expression and subsequent virus incorporation (Robison and Whitt, 2000). However, this cannot explain why RV G chimera D4-E51 was successfully trafficked to the cell surface because this region of the RV G ED does not contain potential glycosylation sites. However, trimerization of the D4-E51 molecules or between wild type RV G and D4-E51 might also be required for transport, a possibility that awaits biochemical analyses.

While we were succeeded in expressing a chimeric RV G/anthrax PA fusion protein on the cell surface, a concern remains regarding transport of the chimeric glycoproteins depending on the size of the foreign polypeptide. However, our preliminary results using the entire PA63 (428 aa) as well as heavy-chain 50 of botulinum neurotoxin A (436 aa) fused to the E51 construct indicate excellent cell surface expression (Koser and Schnell, unpublished). Together, our results show that RV G is permissive for the fusion of large foreign protein fragments, that virus particles can incorporate such a chimeric glycoprotein, and that such RV-based vectors are replication-competent.

It is well accepted that PA is the major the antigenic component of the current anthrax vaccine (Bioport, 2002) and most anthrax vaccines under development focus on PA. The lack of a humoral response to our initial vaccine expressing PA63 by live RV is not entirely surprising since the protein is produced intracellularly and availability to stimulate B cells is therefore probably low. We previously reported that the nucleoprotein of SARS-CoV was not immunogenic in mice when expressed by a similar RV vaccine vector, but high titers of neutralizing antibodies were detected when the SARS-CoV spike protein was expressed by RV (Faber et al., 2005). Even though the reason for this might lie in the different immunogenicity of the two SARS-CoV proteins, it appears that proteins expressed on the cell surface and incorporated into RV virions are more potent inducers of humoral responses than are internally expressed proteins. This notion is supported by the finding that a single immunization with RV induces strong humoral responses against RV G but only modest responses against the internal RV proteins (Foley et al., 2000).

In contrast, PA expressed on the surface of virus particles induced potent humoral responses with either live or killed SPBN-D4-E51 particles stimulating antibody production after only a single dose. Note that several other approaches to anthrax vaccination require multiple doses for induction of antibody responses (Aulinger et al., 2005; Galen et al., 2004; Rhie et al., 2003). This is consistent with our expectation that SPBN-D4-E51 particles maximally stimulate B cells. RV G in wild type virus comprises 24% of the virus mass (Flamand et al., 1993;

Wunner, 1991). The chimeric glycoprotein D4-E51 was incorporated, at best, equivalently to RV G. Thus, attributing 12% of the virus particle mass to the D4-E51 glycoprotein, a dose of 7.5 μ g of SPBN-D4-E51 contains an antigen dose equivalent to 500 ng of rPA. We speculate that this 150-fold decrease in SPBN-D4-E51 dose requirement is due to presentation by the virus particle, consistent with studies comparing the immunogenicity of proteins presented in a soluble form disordered form versus proteins complexed to viruses or virus-like particles (Eckhart et al., 1996; Fehr et al., 1997, 1998).

In addition to maximally stimulating B cells upon prime, the virus particle is expected to be a strong inducer of T cell help, especially as compared to a monomeric recombinant protein. The RV G and nucleocapsid possess CD4+ T helper epitopes (Bunschoten et al., 1989; Ertl et al., 1991; Macfarlan et al., 1984), and exposure of mice to RV ribonucleoprotein significantly augments virus-neutralizing antibodies upon subsequent introduction of RV (Hooper et al., 1994). For anthrax, immunization with PA at antibody-sub-stimulating levels primes for a more robust and rapid humoral response upon boost as compared to without prime (Marcus et al., 2004). As expected, boosting with a second dose of live or killed SPBN-D4-E51 resulted in an increase in anti-PA and anti-RV G titers. High antibody titers also resulted following boost with rPA, but there was a clear difference in the IgG subclass. Antibodies to PA, either induced by vaccination or passively administered, confer protection against challenge (Beedham et al., 2001; Ivins et al., 1998), so that stimulation of a Th2 type response is desirable. Analogous to the case with AVA (Semenova et al., 2004) and with rPA formulated in aluminum hydroxide (Tan et al., 2003) the subclass distribution of sera from mice immunized with 50 ng or more of SPBN-D4-E51 was primarily IgG1 indicating a Th2-biased response. By contrast, the 5 ng dose of SPBN-D4-E51 as well as rPA stimulated Th1-associated antibodies consistent with the notion that IgG subclass response is dependent upon dose and context of antigen presentation.

We have demonstrated that killed RV particles can serve as immunostimulatory carrier molecules for display of foreign B cell antigens. SPBN-D4-E51 recombinant particles were potent inducers of humoral immune responses after even a single inoculation, which might be especially important for pre-exposure treatment, where rapid responses are required. The observed immune responses are Th2-dominated, and immunized mice showed potent memory responses. Our results establish proof-of-concept in the design of the particle and the ability to stimulate B-cell responses. The quality of the antibody response in terms of in vitro protection and challenge awaits subsequent studies.

Materials and methods

Plasmid construction and virus recovery

pGEM PA63 (GenBank accession number DQ190737) contains the sequence encoding the Newcastle disease virus

(NDV) transmembrane (TM) and cytoplasmic domain (CD) followed by the sequence encoding the translocation-deficient form [K397D, D425K (Sellman et al., 2001)] of the 63-kDa fragment of *B. anthracis* PA (anthrax PA, GenBank accession number M22589, (Welkos et al., 1988)). The PA63 gene was optimized for human codon usage to ensure high expression levels. All sequences were synthesized by non-template PCR and inserted into the pGEM-T vector (Promega Inc.) at the *Xba*I site.

The following PCR reactions were performed using VENT polymerase (Biolabs Inc.) and the primers listed in Table 1 (see Supplementary material).

PA63 was PCR-amplified from pGEM PA63 using primers RP187 and RP188. The RV vector pSPBN (Morimoto et al., 2001) and PCR product were digested with *Bsi*WI and *Nhe*I and ligated, resulting in plasmid pSPBN-PA63.

To construct a plasmid encoding PA63 flanked by the RV G signal sequence (SS), CD and TM, the N-terminal 22 amino acids of the RV G including the RV SS (19 aa) was PCR amplified from pTIT-G (McGettigan et al., 2001b) using primers RP77 and RP125 (PCR1). In a second PCR reaction, the sequence encoding RV G TM and CD was amplified from pTIT-G using the primers RP79 and RP80 (PCR2). PCR1 and PCR2 products were digested with *Spe*I and ligated. The ~300 base pair (bp) product was digested with *Xba*I and *Pst*I, and cloned in the *Xba*I- and *Pst*I-digested pBS-SKII+ (Stratagene Inc.). The resulting plasmid was designated pBS-ST5. The PA63 sequence was PCR-amplified from pGEM PA63 using primers RP189 and RP190, digested with *Spe*I and *Hpa*I, and cloned into *Spe*I- and *Hpa*I-cleaved pBS-ST5. The resulting plasmid was designated pPA63-TC. PA63-TC was digested with *Bsi*WI and *Xba*I and cloned into pSPBN, yielding pSPBN-PA63-TC.

Anthrax PA D4 was amplified from pGEM PA63 using primers RP293 and RP310, digested with *Avr*II and *Hpa*I, and cloned into pBS-ST5 to yield pD4-ΔED. To generate the D4-RV G chimeric glycoproteins containing different portions of the RV G ED (see Fig. 3), a series of RV G ED fragments was amplified from pTIT-G using the reverse primer RP299 and forward primers RP295, 296, 297 and 298 and plasmid pTIT-G. The PCR products, as well as plasmid pD4-ΔED, were digested with *Sty*I and *Hpa*I, and the different ED fragments were ligated into pD4-ΔED, resulting in pD4-E439, pD4-E210, pD4-E127 and pD4-E51.

To construct the recombinant RV expressing D4-E51, pD4-E51 and pSPBN were digested with *Bsi*WI and *Nhe*I and the D4-E51 fragment ligated to pSPBN, resulting in pSPBN-D4-E51. All recombinant viruses were recovered using published methods (Foley et al., 2000).

Virus purification and inactivation

BSR cells were infected at a multiplicity of infection (MOI) of 0.1 in Dulbecco's modified Eagle's medium (DMEM, Mediatech, Inc.) supplemented with 10% fetal bovine serum (FBS) and incubated for 2 h with frequent gentle agitation. Cells were washed three times with phosphate-buffered saline

(PBS) and OptiPro medium (Invitrogen, Inc.) was added. Virus was harvested at 3 and 6 days post-infection. Supernatants from the two harvests were pooled and clarified by ultracentrifugation using an SW28 rotor at 22,000 RPM for 1 h at 4 °C. Pelleted material was resuspended in PBS overnight at 4 °C. Beta-propiolactone (BPL, Sigma, Inc.) diluted 1:100 in ice-cold dH₂O was added to the virus solution to a final BPL dilution of 1:2000 and incubation continued overnight at 4 °C. Inactivated virus was then layered over a 20% sucrose gradient and pelleted by ultracentrifugation as described above. The pelleted virus was resuspended in PBS and stored in aliquots at –80 °C.

Virus concentration determination

The concentration of a virus standard was determined by BCA kit (Pierce, Inc.). The BSA standard and the virus were each prepared in detergent solution (final concentration 0.002% NP-40, 0.0007% deoxycholate, 11 mM EDTA, 2 mM Tris–HCl, 0.0002% protease inhibitor, 0.0002% SDS), incubated at 95 °C for 5 min and placed on wet ice immediately prior to analysis. Subsequent lots of virus were analyzed by SDS-PAGE and concentration relative to the virus standard was determined.

Immunostaining

Expression of PA by the viral constructs SPBN-PA63 and SPBN-PA63-TC was analyzed in BSR cells infected at an MOI of 0.01 and incubated for 48 h, while expression of the D4-RV G chimeras was analyzed in BSR cells infected at an MOI of 5 for 2 h with vaccinia virus expressing the T7 promoter (vVT7) (Fuerst et al., 1986) followed by transfection with 5 μg pD4-E439, pD4-E210, pD4-E127, pD4-E51 or pD4-ΔED using calcium phosphate (Stratagene, Inc.). After 2 h incubation, transfected cells were washed twice in DMEM and incubated overnight in DMEM supplemented with 10% FBS. Cells were fixed by incubation in 3% paraformaldehyde for 30 min at room temperature followed by two washes in 10 mM glycine in PBS (PBS-glycine). For internal staining, cells were incubated in 1% Triton X-100 for 5 min at room temperature followed by two washes in PBS-glycine. Cells were immunostained with mouse anti-PA monoclonal antibody M2-V116 (OEM, Inc.) followed by FITC-labeled donkey anti-mouse IgG (Jackson ImmunoResearch, Inc.) or FITC-labeled anti-RV nucleoprotein (Centocor, Inc.).

Fluorescence-activated cell sorting (FACS) analysis

BSR cells were transfected with Bluescript SKII (Stratagene, Inc.), pD4-E439, pD4-E210, pD4-E127, pD4-E51, or pD4-ΔED as described above, washed once with PBS, detached from the plate by treatment with 50 mM EDTA for 5 min at room temperature, and washed twice in FACS buffer (2% FBS in PBS). Cells were incubated with a 1:200 dilution of anti-PA antibody M2-V116 in FACS buffer for 1 h at room temperature, washed twice in FACS buffer, and further incubated with a

1:200 dilution of FITC-labeled donkey anti-mouse IgG (Jackson ImmunoResearch, Inc.). After two washes in FACS buffer, cells were fixed in formaldehyde (Caltag GAS004 Fix and Perm) for 15 min at room temperature, washed twice in FACS buffer and analyzed by FACScan (Beckman Coulter XL analyzer with a 488-nm argon ion laser).

SDS-PAGE and western blotting

BSR cells transfected overnight with plasmids pD4-E439, pD4-E210, pD4-E127, pD4-E51 or pD4- Δ ED as described above, were washed twice with PBS and incubated with lysis buffer (1% NP-40, 0.4% deoxycholate, 66 mM EDTA, 10 mM Tris-HCl, protease inhibitor, 0.1% SDS) for 5 min on wet ice. Lysates and purified virus were prepared for Western analysis by adding protein-loading solution to a final concentration of 2% beta-mercaptoethanol and heating for 5 min at 95 °C. Proteins were separated by 8% SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane (Osmonics, Inc.). Blots were blocked with 5% low-fat milk powder in PBS followed by incubation with either a 1:2000 dilution of anti-PA M2-V116 or a 1:25,000 dilution of anti-RV G CD (Siler et al., 2002) in PBS for 1 h at room temperature, and washed twice in 0.1% Tween-20 in PBS (PBS-tween). Blots were probed with a 1:5000 dilution of HRP-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch, Inc.) or donkey anti-rabbit IgG (Jackson ImmunoResearch, Inc.) and washed twice as described above. Chemiluminescence was applied as instructed by the manufacturer (Sigma, Inc.).

Preparation of antigens for ELISAs and lymphocyte proliferation assay

To generate RV G, virus was purified as described above. Octyl β -D-glucopyranoside (Sigma) was added to a final concentration of 1%. The material was centrifuged at 100,000 \times g at 15 °C for 1 h. RV G contained in the supernatant was removed, diluted to 1 mg/ml in PBS and stored at -70 °C. Viral ribonucleoprotein (RNP) was prepared as described (Koser et al., 2004). All antigens were quantified by BCA kit according to manufacturer's protocol (Pierce). Recombinant PA was obtained from the Biodefense and Emerging Infections Research Resources Repository (*beiresources*).

Immunization of mice

For comparison of live and killed vaccines, groups of five 6- to 8-week-old Swiss Webster mice were immunized intramuscularly (i.m.) in the gastrocnemius with 50 μ g of inactivated SPBN-D4-E51, 3×10^6 focus-forming units (ffu) of live SPBN-D4-E51, 50 μ g of inactivated SPBN, or 3×10^6 ffu of live SPBN. Mice were boosted as for primary inoculation 3 weeks after first immunization and blood samples were obtained at 2 weeks after each inoculation.

For dose-response studies, groups of three 6- to 8-week-old Swiss Webster mice were immunized i.m. in the gastrocnemius

with 50 μ g, 5 μ g, 500 ng, 50 ng or 5 ng of inactivated SPBN-D4-E51 or with 100 ng RV G + 100 ng rPA (List Biological Laboratories, Inc.). Mice were boosted 3 weeks later and bled 2 weeks after each inoculation. In a second experiment, mice were immunized as above with the same doses of inactivated SPBN-D4-E51 or with 500 ng RV G + 500 ng rPA.

For lymphocyte proliferation analysis, groups of four 6- to 8-week-old Swiss Webster mice were either left unimmunized or immunized i.m. in the gastrocnemius with 50 μ g of inactivated SPBN-D4-E51 on days 0 and 21. At 4 weeks post-boost, mice were sacrificed and spleens harvested.

All animal experiments were performed under IACUC-approved protocols (Animal Welfare Assurance No. A3085-01).

ELISAs

ELISA plates (96-well) were coated with 200 ng/well of rPA (*beiresources*) or 100 ng/well RV G in coating buffer (5 mM Na₂CO₃, pH 9.6) overnight at 4 °C. Plates were washed four times in PBS-tween and blocked with 5% low-fat milk in PBS for 1 h at room temperature. 100 μ l sera diluted in PBS as indicated in the figures were added to wells and incubated for 1 h at room temperature. After washing plates 4 times in PBS-tween 100 μ l HRP-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch, Inc.) diluted was added per well and incubation continued at 37 °C for 30 min. Plates were washed four times with PBS-tween before OPD substrate prepared according to the manufacturer's instructions (Sigma, Inc.), was added. Incubation was continued for 15 min at room temperature in the dark and the reaction was stopped by adding 2 M H₂SO₄. Optical density at 490 nm was determined.

Gamma-globulin subclass-specific ELISAs were performed using one dilution series prepared for each of the three mice and distributed to duplicate plates. Assays were performed as above, using the secondary antibodies IgG1 (Southern Biotechnology, Inc.) and IgG2a (Southern Biotechnology, Inc.) at 1:1500 and 1:1000 dilutions, respectively.

Lymphocyte proliferation assay

Single-cell suspensions from mouse spleens harvested 4 weeks post-boost were prepared by homogenization and passage through a 100- μ m cell strainer. Erythrocytes were lysed and cells were washed and diluted in Iscove's modified DMEM supplemented with 5% FCS. For each mouse, 2.5×10^6 cells/well were seeded in 24-well plates. Cells in triplicate wells were stimulated with 0.3 μ g RV G, 1 μ g RV RNP, 1 μ g SPBN or 10 μ g rPA. One set of wells without antigen served as the medium control. At various time points, 100 μ l of cells was removed to a 96-well plate and pulsed with 1 μ Ci of [3H]-thymidine. Cells were harvested 24 h later onto glass fiber filter mats using a cell harvester (Skatron), placed in scintillation vials with 3 ml scintillation fluid and assessed for radioactivity in a scintillation counter. Average from triplicate wells was calculated in counts per minute (CPM) and CPM of the medium control subtracted for each antigen.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.virol.2006.05.010](https://doi.org/10.1016/j.virol.2006.05.010).

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