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A replication-incompetent Rift Valley fever virus: Chimeric virus-like particles protect mice and rats against lethal challenge

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Virus-like particles (VLPs) present viral antigens in a native conformation and are effectively recognized by the immune system and therefore are considered as suitable and safe vaccine candidates against many viral diseases. Here we demonstrate that chimeric VLPs containing Rift Valley fever virus (RVFV) glycoproteins G0 and GC, nucleoprotein N and the gag protein of Moloney murine leukemia virus represent an effective vaccine candidate against Rift Valley fever, a deadly disease in humans and livestock. Long-lasting humoral and cellular immune responses are demonstrated in a mouse model by the analysis of neutralizing antibody titers and cytokine secretion profiles. Vaccine efficacy studies were performed in mouse and rat lethal challenge models resulting in high protection rates. Taken together, these results demonstrate that replication-incompetent chimeric RVF VLPs are an efficient RVFV vaccine candidate.

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Introduction

Rift Valley fever virus (RVFV) is a devastating mosquito-borne viral zoonotic disease that causes serious morbidity and mortality in both humans and livestock. In ruminants, RVF is characterized by substantial mortality of young animals (especially of lambs), fetal deformities and abortion (Flick and Bouloy, 2005; Gerdes, 2004; Swanepoel and Coetzer, 2003). In humans the disease is often associated with benign fever but can lead to more complicated cases such as retinal vasculitis, encephalitis, neurologic deficits, hepatic necrosis, or fatal hemorrhagic fever (Flick and Bouloy, 2005; Geisbert and Jhariing, 2004; Meegan, 1979). Interestingly, human case fatality rates increased significantly during the last several years. While historically less than 2% of infected individuals developed a fatal hemorrhagic fever, analysis from recent outbreaks (2007/2008) reveal a 20–30% fatality rate in humans (LaBeaud et al., 2008). However, differences in case definition, accuracy in disease surveillance methods and data gathering methodology likely impact these numbers.

RVFV is a member of the Bunyaviridae family, which includes more than 300 viruses grouped into five genera (Orthobunyavirus, Hanta-

virus, Nairovirus, Phlebovirus, and Tospovirus). Bunyaviruses are enveloped viruses with a tripartite, single-stranded RNA genome of negative and sometimes ambisense polarity (Elliott, 1996; Elliott et al., 1991; Schmaljohn and Hooper, 2001). The large (L) genomic RNA segment encodes the RNA-dependent RNA polymerase (L), the medium (M) segment the glycoprotein precursor, which is post-translationally processed into the two mature spike proteins (G1 and G2, or by the new convention: G0 and GC), and in some viruses a non-structural protein (NSM), while the small (S) segment encodes the nucleoprotein (N), and in some viruses a non-structural protein NS2 (Elliott, 1996; Schmaljohn and Hooper, 2001).

RVFV has traditionally caused recurrent outbreaks affecting humans and ruminants predominantly in Sub-Saharan Africa, but spread to Egypt in 1977 and to the Arabian Peninsula in 2000 (Al-Hazmi et al., 2003; Anonymous, 2000; Balkhy and Memish, 2003; Madani et al., 2003; Shoemaker et al., 2002). More recently, RVFV circulated in East Africa causing serious epidemics in Kenya, Tanzania, Somalia, Sudan and was reported in the Comoros Islands (LaBeaud et al., 2008; WHO, 2007) in 2007, and subsequently expanded to Madagascar and South Africa in 2008 [http://www.fao.org/docs/eims/upload/242253/EW_rvf_apr08.pdf].

RVFV is a prototype of emerging/re-emerging pathogens and is classified as a Category A High Priority Pathogen by the National Institute for Allergy and Infectious Diseases (NIAID) [http://www3.niaid.nih.gov/topics/BiodefenseRelated/Biodefense/research/CatA.}
polyomavirus, Bluetongue virus, rotavirus, retroviruses, bunyaviruses, hepatitis B virus (HBV), human papillomavirus (HPV), Norwalk virus, human immunodeficiency virus (HIV), and human papillomavirus (HPV). The lack of prophylactic and therapeutic measures, the potential for human-to-human transmission, and the significant threat to livestock associated with RVFV make infection with this pathogen a serious public health concern not only in endemic, developing countries, but also in many non-endemic developed countries due to recent bioterror threats, and clearly illustrates the need for more RVFV vaccine research and development.

MP12, a highly attenuated (by 5-fluorouracil treatment in cell culture) human virus isolate of RVFV (Caplen et al., 1985; Vialat et al., 1997), has recently been tested in a phase II safety/efficacy clinical trial (ClinicalTrials.gov identifier: NCT00415051) to determine if it is safe to give to humans (results not yet published). MP-12 also has potential veterinary applications (Hunter et al., 2002).

A formalin-inactivated RVFV vaccine, TSI-GSD-200, has been developed; however, it is not licensed and not commercially available (Pittman et al., 1999). TSI-GSD-200 is only provided to veterinarians working in endemic areas, high containment laboratory workers and others at high risk for contracting RVFV (Pittman et al., 1999). Unfortunately, this vaccine is (i) expensive, (ii) difficult to produce, (iii) in short supply, (iv) requires larger dose relative to an attenuated vaccine and three initial inoculations followed by a 6-month booster (v) and requires continued annual boosters to maintain protective immunity (Frank-Peterside, 2000; Kark et al., 1982; Kark et al., 1985; Niklasson et al., 1985).

The use of virus-like particles (VLPs) is a promising approach for the development of a safe and efficient RVFV vaccine. Expression of structural proteins of many non-enveloped and enveloped viruses leads to the formation of VLPs (Garcea and Gissmann, 2004; Grigacic and Anderson, 2006a, 2006b; Noad and Roy, 2003). Such VLPs frequently exhibit a morphology very similar to that of wild-type (wt) viruses (Johnson and Chiu, 2000). Since VLPs have a tropism similar to that of the wt virus and show comparable cellular uptake and intracellular trafficking, the formation of VLPs can be used to study virus assembly and morphogenesis, budding processes, genome packaging, receptor binding, and virus entry (Bos et al., 1997; Johnson and Chiu, 2000; Li et al., 2003; Licata et al., 2004; Overby et al., 2006; Schmitt et al., 2002; Ye et al., 2006). Especially for viruses classified as high containment agents, e.g., RVFV and Ebolavirus, the development of VLP systems are of practical use because subsequent work can be performed under lower biosafety conditions (Habjan et al., 2009; Naslund et al., 2009; Warfield et al., 2004; Watanabe et al., 2004). VLPs present viral antigens in a native conformation and are effectively recognized by the immune system (Grigacic, 2006; Grigacic and Anderson, 2006; Noad and Roy, 2003).

Many promising vaccine candidates based on VLPs are at various stages of development, including vaccine candidates for hepatitis B virus (HBV), human papillomavirus (HPV), Norwalk virus, human poliovirus, Bluetongue virus, rotavirus, retroviruses, bunyaviruses and filoviruses (Garcea and Gissmann, 2004; Grigacic, 2006; Habjan et al., 2009; Naslund et al., 2009; Noad and Roy, 2003).

These promising attempts to generate VLP-based vaccines against many different animal and human pathogens encouraged us to evaluate RVF VLPs as vaccine candidates against RVFV. Here we describe the generation of chimeric RVF VLPs, a novel concept for bunyaviruses, the optimization of VLP production and their successful use as vaccine candidates. Vaccine efficacy was analyzed through immunological studies of vaccinated mice and in lethal challenge studies in two different rodent models. High protection rates and robust and long-lasting immune response of vaccinated animals demonstrate that chimeric RVF VLPs are a promising approach to generate safe and efficient RVFV vaccines.

**Results**

**Generation and characterization of RVF VLPs**

RVF VLPs were generated by transient transfection of HEK-293 or 293-gag cells (HEK-293 cells that constitutively express Moloney murine leukemia virus (MoMLV) gag protein) with expression plasmids encoding the RVFV glycoproteins and the nucleoprotein (N). We initially focused upon the generation of chimeric (MoMLV gag-containing VLPs, designated RVF chimVLPs) because it has been previously shown that the inclusion of retroviral gag can increase the uniformity and quantity (Gheysen et al., 1989; Halfar et al., 1990; Haynes et al., 1991; Rovinski et al., 1992; Szecsi et al., 2006) and stability (Hammonds et al., 2003) of VLPs. In addition, MoMLV gag protein could have an adjuvant-like effect. RVF VLPs were harvested

![Fig. 1. Characterization of RVF VLPs. (A) Western blot analysis of chimeric RVF VLPs (chimVLP): concentrated supernatants from 293-gag cells transfected with RVFV G and N expression plasmids analyzed by Western blotting using antibodies specific for RVFV G\_G, G\_C, N and Moloney murine leukemia virus (MoMLV) gag. (B) Western blot analysis of RVF VLPs: Concentrated supernatants from 293 cells transfected with RVFV G and N expression plasmids analyzed by Western blotting using antibodies specific for RVFV G\_G, G\_C, N and Moloney murine leukemia virus (MoMLV) gag. (C) Negative staining of RVFV G and N transfected 293 cells fixed with glutaraldehyde and stained with uranyl acetate and examined by transmission electron microscopy. Scale bar represents 200 nm. Left panel: arrows point to budding VLPs; right panel: arrows indicate RVFV G spikes protruding from the VLP membrane. (D) Western blot analysis of RVF chimVLPs: Concentrated supernatants from 293-gag cells transfected with RVFV N and wild type (WT) or codon-optimized (CO) RVFV G sequences.**
from tissue culture supernatants from 48 to 96 h post-transfection, concentrated by tangential flow filtration and purified by ultracentrifugation through a 20% sucrose cushion. To analyze VLP components, samples were fractionated by SDS-PAGE and analyzed by Western blots using RVFV and MoMLV-specific antibodies.

Specific signals were detected confirming the presence of RVFV GN, GC and N in 293 and 293-gag cell-derived VLP preparations (Fig. 1A, first three panels and Fig. 1B), as well as MoMLV gag in the chimeric VLP (chimVLP) preparation derived from 293-gag cells (Fig. 1A, right panel). Note that the gag species are detected as both precursor (arrow 1) and mature, proteolytically processed species (arrows 2 and 3) (Suomalainen et al., 1996; Yoshinaka et al., 1985).

Transmission electron microscopy was used to analyze the structure of the generated VLP preparations, demonstrating their uniform nature and size. Newly synthesized VLPs are formed at intracellular membranes, presumably the Golgi apparatus (based on studies of bunyavirus glycoproteins; Andersson et al., 1997; Gerrard and Nichol, 2002; Haferkamp et al., 2005; Shi and Elliott, 2007), and bud into vesicles analogous to RVF virions (Fig. 1C, left panel). RVFV glycoprotein spikes embedded in the VLP membranes are visible (Fig. 1C, right panel, arrows).

We next attempted to optimize RVF VLP production by using expression plasmids containing a codon-optimized RVFV G gene. Suboptimal codon usage often leads to the inefficient expression of viral protein genes in mammalian cells (Barrett et al., 2006; Haas et al., 1996; Zhou et al., 1999). We therefore compared the amount of RVF chimVLPs generated by 293-gag cells transfected with expression plasmids encoding either native or codon-optimized (Babcock et al., 2004) RVFV G genes. No significant difference in GN content of concentrated and purified VLP preparations was observed when separated by SDS-PAGE and analyzed by Western blot (Fig. 1D). However, the use of the codon-optimized RVFV G appears to have a minimally beneficial effect on VLP yield. Similar results were obtained when RVF VLPs were generated in 293 cells (data not shown). Therefore, for all subsequent experiments the codon-optimized sequence was utilized.

To further characterize the individual components of the RVF VLPs, sucrose density gradient fractionation was employed. This technique has been successfully used in other studies to elucidate the specific components in VLP preparations (Haynes et al., 2009; Ye et al., 2006; Young, Smith, and Ross, 2004). The basis for this approach is that chimVLPs are of a different density than VLPs based on the different protein composition. Results suggest that RVFV GN and MoMLV gag are located within the same particle in the generated chimVLPs (data not shown). Subsequent immunoprecipitation experiments with RVFV GN antibodies and protein A/G-coated agarose beads confirm these results (data not shown).

![Fig. 2. Optimization of RVF VLP production. (A) Different plasmid ratios to determine optimal chimeric RVF VLP production: 293-gag cells were transfected with 15, 9 or 3 μg of the RVFV G expression plasmid, and co-transfected with 0, 3, 9 or 15 μg RVF N expression plasmid as indicated. Concentrated supernatants of a 60 h post-transfection harvest were analyzed by Western blot using antibodies specific for RVFV GN. (B) Time course experiment to optimize RVF VLP yields: Western blot analysis was performed using antibodies specific for RVFV G and GC, as indicated at each blot of RVF chimVLPs and VLPs harvested at select times post-transfection. Densitometric analysis of band intensity, displayed as % maximum band intensity for a particular blot, is represented by histograms above each Western blot. Harvest times are indicated.](fig2.png)

![Fig. 3.Neutralizing antibody titer in RVF VLP-vaccinated mice determined by plaque reduction neutralization tests. Mouse sera were collected after three immunizations with RVF chimVLPs, RVF VLPs with or without RVFV N, Ebolavirus GP-pseudotyped MoMLV (Control 1) and from unimmunized mice (Control 2). The neutralizing antibody titer was determined as the reciprocal of the dilution of five two-fold serial dilutions of sera, respectively. Neutralizing antibody titer is considered positive at the lowest initial serum dilution that results in >80% (PRNT<sub>80</sub>) reduction of the number of plaques as compared to the virus control.](fig3.png)
Optimization of RVF VLP generation

Generation of VLP-based vaccine candidates for RVFV from mammalian cells is labor-intensive and costly in regard to both time and reagents. Therefore it is critical to perform the necessary process development to establish efficient manufacturing procedures. Therefore, additional optimization experiments were performed to maximize RVF VLP production in adherent mammalian cells.

Different amounts of expression plasmids were transfected in the first step to optimize VLP production. Three different amounts (3, 9 and 15 μg) of the RVFV G expression plasmid were used and analyzed with increasing amounts of RVFV N expression plasmid (from 0 to 15 μg). RVF chimVLPs were harvested at 12-h intervals post-

Fig. 4. Antigen-specific cytokine secretion by splenocytes of chimeric RVF VLP-vaccinated mice measured by multiplex analysis. Tissue culture supernatants from splenocytes harvested at 24, 96 or 168 h post-antigen stimulation from mice immunized with RVF chimVLPs, influenza VLPs or an unvaccinated mouse were subjected to multiplex bead analysis to measure select cytokines. Time of harvest and the cytokine measured are described at the top and left of the panels, respectively. Data from splenocytes harvested from the control mouse are indicated by red bars, and data from RVF chimVLP-vaccinated mice are indicated by blue bars. Stimulatory antigens are indicated below the graphs. Insets show the same results as the main graphs except with reduced scales (Y-axis, pg/ml) to show that secretion is detected at many points but is masked by the scale required to illustrate maximal cytokine signal.
transfection, purified and concentrated as described above and fractionated by SDS-PAGE. RVF chimVLP generation was measured by Western blot analysis using antibodies specific for RVFV Gn.

As shown in Fig. 2A, generation of RVF chimVLPs is strongly affected by the amount of the RVFV G- and N-encoding expression plasmids used for transfection of 293-gag cells. A high amount (≥ 9 μg) of RVFV N expression plasmid combined with a lower amount of RVFV G expression plasmid (3 μg) (molar ratio RVFV N:G = 4:3) resulted in the best VLP yields (as indicated by Gn signal). These conditions also resulted in the most detectable RVFV N in the VLP preparations (data not shown). Interestingly, this analysis also shows that RVF VLPs can be generated without inclusion of the N expression plasmid by transfection of RVFV glycoprotein expression plasmids into 293 cells (Fig. 2A, lanes 4, 8 and 12).

Previous studies demonstrate that the inclusion of a minigenome encoding a reporter gene can be a useful tool for the determination of VLP titer and to potentially increase the N content in VLPs (Handajen et al., 2009; Overby et al., 2006). Therefore, an M segment-based minigenome under the control of a RNA polymerase I promoter (Billecocq et al., 2008) was co-transfected in concert with the RVFV G and N expression plasmids to determine if encapsidation of this minigenome with RVFV N and subsequent packaging into the budding VLPs could increase overall N content. However, no significant change in either VLP yields or N content was observed (data not shown).

Next, the optimized transfection scheme (3 μg RVFV G and 9 μg RVFV N expression plasmids) was used for the determination of the optimal times to harvest RVF chimVLPs post-transfection to obtain the best yields. Focusing the time of harvest to a shorter interval will clearly increase the VLP titer and to potentially increase the N content in VLPs (Habjan et al., 2008). As shown in Fig. 2B, generation of RVF chimVLPs is strongly affected by the amount of the RVFV G- and N-encoding expression plasmids (chimVLP), VLPs produced in 293 cells with RVFV G and N (VLP + N) and VLPs produced in 293 cells without RVFV N (VLP - N). Six harvests, at 24, 36, 48, 60, 72 and 84 h post-transfection will recover most of the RVF chimVLPs or RVF chimVLPs produced. After optimizing expression strategies, transfection conditions (data not shown), plasmid amounts, plasmid ratios and harvest times, we are able to generate sufficient VLP material for subsequent studies.

Immunogenicity of RVF VLPs

We next addressed the question of whether the generated RVF VLPs can be used as a replication-incompetent vaccine candidate. We first analyzed the immune response in mice (neutralizing antibody titers, durability and cytokine expression levels) induced by RVF VLPs and subsequently performed protection studies in two different rodent models.

Viral vaccine efficacy often correlates with seroconversion – specifically the generation of virus-neutralizing antibodies (Khanam et al., 2006; Ye et al., 2006). Therefore, we first performed durability studies to determine whether RVF VLPs induce long-lasting RVFV neutralizing antibodies. Mice were immunized subcutaneously (s.c.) three times with RVFV chimVLPs at 9-day intervals, and blood was collected 161 days post 3rd vaccination (179 days post first vaccination). Plaque reduction neutralization PRNT 80 assays (Mangiafico et al., 1988) using RVFV ZH501-infected VeroE6 cells were performed by combining serum with 60 pfu ZH501 virus. The neutralizing antibody titer is considered positive at the highest serum dilution that inhibits 80% of the plaques compared with the virus control titration. As shown in Fig. 3, neutralizing titers of >1:640 were obtained for all RVF chimVLP and RVF VLP - N (no nucleoprotein) vaccinated mice (n = 4), while three of four RVF VLP + N-vaccinated mice developed titers of >640 and one of 320. No seroconversion was detected in a control group vaccinated with an unrelated MoMLV-based vaccine (Ebolavirus GP-pseudotyped MoMLV; Control 1) or non-vaccinated mice (Control 2). Such high neutralizing titers observed more than 6 months post-vaccination indicates a durable RVFV vaccine candidate and warrants further evaluation of these RVF VLP-based vaccine candidates.

Next, we studied antigen-specific secretion of select cytokines by splenocytes isolated from vaccinated mice to determine whether RVF VLP-based vaccines elicit both humoral and cellular immunity. These responses have been correlated with vaccine efficacy in live challenge models (Wack et al., 2008). Importantly, anti-viral immunity often correlates with the development of cellular immune response (Warfield et al., 2005a). Splenocytes were harvested from mice vaccinated three times with RVF chimVLPs (days 0, 9 and 18) 31 days post final vaccination and cultured in 24-well dishes. Cells were stimulated by the addition of heat-inactivated live attenuated RVFV strain MP12 (Caplen et al., 1985) or a control antigen, heat-inactivated influenza A virus strain A/HK/1/68. Supernatant samples were taken at 24, 96, and 168 h and subjected to cytokine analysis using a bead-based multiplex system (Bioplex, BioRad). Splenocytes from vaccinated mice secrete antigen-specific cytokines in response to MP12 stimulation, but not to the unspecific influenza antigen (Fig. 4, blue bars). Splenocytes from the control (unvaccinated) mouse do not respond to either stimulus (Fig. 4, red bars). IL-2, IL-4, IL-5 and IFN-γ production is elicited by the RVFV-specific antigen, consistent with both humoral (Tn12) and cellular (Tn1) responses (Chung et al., 2008; Fromantin et al., 2001). Interestingly, cytokine levels peak at different times post-stimulation, which suggests that antigen-dependent expansion of T cells is occurring in vitro.

RVF VLP vaccine efficacy studies

While the immunological data (neutralizing antibody titers and cytokine secretion levels) suggests the tested RVF VLP-based vaccine candidates are immunogenic, these immune correlates alone are not always predictive of efficacy in a live challenge model. Therefore, groups of 20 mice were immunized on days 0, 9, and 18 with select vaccine candidates: chimeric VLPs produced in 293-gag cells (chimVLP), VLPs produced in 293 cells with RVFV G and N (VLP + N) and VLPs produced in 293 cells without RVFV N (VLP - N). Forty-six days after the third vaccination (64 days post first vaccination), 16 of 20 animals were challenged with 106 pfu RVFV ZH501 under biosafety level 4 (BSL-4) conditions and observed for signs of disease for 18 days (Fig. 5). The remaining four mice per group were used for the PRNT 80 experiments (Fig. 3). As expected, all animals vaccinated with PBS only or a non-specific MoMLV-based vaccine (control 1 and 2, respectively) succumbed to RVFV challenge within the first 4 to 6 days. This demonstrates that MoMLV-specific components of the vaccines do not protect mice from RVFV challenge. The survival rate is 0/16 for RVFV vaccine candidates. Nine of 16 (56%) of
the VLP + N vaccinated mice survived lethal challenge, while only three of 16 (~19%) survived if immunized with VLP - N vaccine. These results show that protection from RVFV challenge is dependent upon the presence of RVFV G proteins and is enhanced by the presence of RVFV N. chimVLP-vaccinated mice showed the best survival rates, as 11 of 16 (68%) mice survived the lethal challenge dose. Significance was determined using the Mantel–Cox test, and significant differences exist between groups including chimVLP and VLP - N (P = 0.001), VLP + N and VLP - N (P = 0.0196), all three vaccinated groups and controls 1 and 2 (P = 0.0001 (chimVLP), P = 0.0001 (VLP + N) and P = 0.0007 (VLP - N). There is no significant difference between the chimVLP and VLP + N groups (P = 0.393).

Rats present another important alternative animal model for RVFV disease and are often considered more relevant than mice (Anderson and Peters, 1988; Anderson et al., 1991b; Bird, Albarino, and Nichol, 2007). Therefore, after promising data were obtained in the mouse model, RVF VLP-based vaccine efficacy (survival) was next analyzed in a lethal rat challenge model. Our objective was to test only the best vaccine candidate (based on the immunological and mouse protection studies) with a minimum number of animals. Groups of six rats were immunized three times at 2-week intervals with the chimeric RVF VLP vaccine candidate or a sterile saline control. Sixty-seven days post first vaccination, rats were challenged with a high dose of 10^5 pfu RVFV ZH501 and examined for signs of disease for 16 days. Body weight was monitored daily as an indication of the overall health of the rats. As shown in Fig. 6A, while all control rats succumbed to disease by day 4 post-challenge, the RVF chimVLP vaccine candidate protected 100% of the vaccinated rats. Unvaccinated control rats showed rapid and substantial weight loss before succumbing to challenge, whereas RVFV chimVLP-vaccinated animals generally maintained their weight throughout the course of the experiment (Fig. 6B).

Fig. 6. RVF VLP efficacy studies in rats. Results are shown as Kaplan–Meier survival curves and weights of VLP-immunized rats after RVFV challenge. (A) Rats (n = 6) were inoculated with the RVF chimVLP vaccine candidate and then challenged with 1 × 10^5 pfu of RVFV strain ZH501. Control rats were immunized with sterile saline. (B) The mean and standard deviation of the weight change for RVF chimVLP vaccinated (circles) and control (squares) rats are shown for each time point.

Overall, immunological as well as lethal challenge studies in two different rodent models clearly demonstrate that RVF VLP-based vaccines are a promising concept for the development of a vaccine for use in humans and livestock. Additional experiments to determine minimum vaccine dose and vaccination schedule as well as safety and efficacy studies in non-human primates are required to further evaluate this promising vaccine platform.

Discussion

The structural proteins of many viruses can assemble into VLPs (Grgacic and Anderson, 2006). VLPs are often described as being more efficacious in the activation of immune responses than conventional protein immunogens/subunit vaccines because their immunogenic protein components are displayed in a high density, more authentic conformation, often with intact biochemical functionality that is effectively immunogenic (Garcea and Gissmann, 2004; Grgacic, 2006; Noad and Roy, 2003). This is further enhanced by the particulate nature of VLPs that appears to be optimal for uptake by dendritic cells (Fifs et al., 2004). In addition, they are safer than inactivated and attenuated virus vaccines because they are usually free of viral genetic material and therefore are not encumbered by the possible safety-related drawbacks including reversion, recombination and re-assortment. At this stage, the ability to develop VLPs does not appear to be limited to any one type of virus or virus family, or by the complexity of the virus particle.

Mimicking the structure of virus particles allows the use of lower doses of antigen to elicit a similar protective response when compared to subunit vaccines (Noad and Roy, 2003). In addition to their ability to stimulate B-cell-mediated immune responses, VLPs have been shown to be highly effective at stimulating CD4 proliferative and cytotoxic T lymphocyte responses (Murata et al., 2003; Paliard et al., 2000; Schirmbeck et al., 1996). This feature of VLP-based vaccines likely plays a major role in their effectiveness as vaccines against viral diseases. The well-documented immunogenicity of VLPs is likely facilitated by their interaction with dendritic cells (Warfield et al., 2003).

Several VLP-based vaccines are in human clinical trials or are FDA-approved, including those for hepatitis B virus (Andre and Safary, 1987; McAleer et al., 1984; Sitrin et al., 1993), trivalent influenza H1N1, H3N2 and B vaccine (“FluBlok”) (Cox et al., 2008; Treanor et al., 2007), H5N1 “bird flu” (Perrone et al., 2009), human papillomavirus (2007; Giannini et al., 2006; Harper et al., 2004; Harro et al., 2001; Joura et al., 2007), human immunodeficiency virus (Young et al., 2006) and Norwalk virus (Tacket et al., 2003). Other VLP-based vaccines with very promising pre-clinical results include VLPs for the severe acute respiratory syndrome (SARS) coronavirus (Lokugamage et al., 2008), human polyomavirus (Goldmann et al., 1999), rotavirus (Clarlet et al., 1998; El-Attar et al., 2009; Jiang et al., 1999), and Ebola and Marburg viruses (Swenson et al., 2008; Swenson et al., 2005; Warfield et al., 2003; Warfield et al., 2005b; Warfield et al., 2007). Additionally, VLP vaccines also have important agricultural applications, including promising vaccines for livestock diseases including bluetongue (Noad and Roy, 2003; Roy, 2000; Roy et al., 2009; Roy and Noad, 2008) and foot-and-mouth (Li et al., 2008; Remond et al., 2009).

Few studies have been undertaken to develop VLPs for bunyaviruses. Bunyamwera VLPs were generated by co-expression of GN, GC and NSm, in addition to a minigenome system (bunyamwera L, N and minigenome) in mammalian cells and were used to identify viral protein components required for virus assembly (Shi et al., 2009; Shi et al., 2006; Shi et al., 2007). Similarly, it has been shown that the expression of recombinant GN and GC glycoproteins of Uukuniemi (UUK) virus, a phlebovirus closely related to RVFV, leads to the assembly and budding of VLPs from transfected mammalian cells (Overby et al., 2007a; Overby et al., 2006; Overby et al., 2007a, 2007b). These VLPs are similar in structure to wt virus and are neutralized by UUK-specific antibodies. However, no immunological in vivo studies were performed to determine immunogenicity.
Generation of RVFV VLPs in insect cells has been demonstrated by Liu et al. (2008) using a single recombinant baculovirus that expresses the RVFV glycoproteins (Gn/Gc) and the N protein (Liu et al., 2008). Efficient generation of RVF VLPs in mammalian cells has been recently demonstrated by Habjan et al. (2009) using transfected DNA encoding the complete RVFV M segment as well as the RNA polymerase I, the nucleoprotein N and a GFP-expressing minigenome (Habjan et al., 2009; Naslund et al., 2009). Näsland et al. (2009) showed that these RVF VLPs can be used for vaccine studies. Three intraperitoneal injections of 1 x 10^6 RVF VLPs in mice induces antibody titers from 1:300 to 1:900 against Gn and Gc proteins but does not result in the development of detectable N-specific antibodies. Importantly, these VLPs protect 11 of 12 vaccinated mice from lethal virus challenge (2.4 x 10^4 pfu), whereas only 1 of 12 survived in the unvaccinated control group (Naslund et al., 2009).

The generation of chimeric RVF VLPs and its successful use as a vaccine candidate is unique to the field of bunyaviruses. We have established an efficient system to generate RVFV chimVLPs and VLPs from 293-gag and 293 cells (Figs. 1 and 2). These VLPs are immunogenic as indicated by the development of neutralizing antibodies in vivo by immunized mice (Fig. 3) and antigen-specific secretion of immune-related cytokines by splenocytes from vaccinated mice (Fig. 4). Furthermore, these VLP-based vaccine candidates are partially protective in mice and 100% protective in rats against lethal challenge (Figs. 5 and 6). Interestingly, RVF VLP production requires only the expression of the two glycoproteins Gn and Gc, and therefore RVFV N is not required. While consistent with findings by Overby et al. (2006) who were able to generate UUK and bunyamwera VLPs without N, this contradicts recent findings that suggest RVF VLPs could only be generated through expression of RVFV glycoproteins together with RVFV N (as part of the minireplicon system) (Habjan et al., 2009). A possible explanation for this discrepancy is that the use of different expression systems (e.g., chicken β-actin vs. immediate-early cytomegalovirus promoter) leads to substantially different amount of RVFV G being produced, and RVF VLPs derived only from the expression of RVF G requires high levels of expression.

The generation of RVF VLPs lacking the N protein facilitates the rational vaccine design for the generation of a safe and highly efficient RVFV vaccine following the DIVA (Differentiating Infected from Vaccinated Animals) concept to differentiate vaccinated from infected individuals (see Bird et al., 2008; Capua et al., 2004). Because RVFV-specific antibodies against the N proteins are easily detected in infected individuals, a vaccine candidate lacking the N antigens facilitates DIVA. However, further studies have to be performed (e.g., increased vaccine dose, different adjuvants) to increase N-lacking VLP vaccine efficiency (see Fig. 5).

chimVLPs containing a retroviral gag protein (either MoMLV or simian immunodeficiency virus (SIV) gag) and the antigen of interest (e.g., influenza hemagglutinin and neuraminidase) have been recently described (Guo et al., 2003; Haynes et al., 2009). Unfortunately, the generation of RVF chimVLPs is more complicated because RVFV G and MoMLV gag localize to the Golgi (Gerrard and Nichol, 2002; Schmaljohn and Hooper, 2001; Wasmoen et al., 1988) and plasma membranes (Soneoka et al., 1997), respectively, in mammalian cells. However, over-expression of RVFV G leads to some Gn/Gc localization at the cell surface (Filone et al., 2006; Gerrard and Nichol, 2002; Gerrard and Nichol, 2007; Liu et al., 2008), which allows the generation of RVF chimVLPs. Further attempts to increase RVFV G surface localization by generating chimeric RVFV G proteins containing the ectodomain of RVFV G and the transmembrane domain and cytoplasmic tail of the MoMLV Env (C-terminal 56aa of the envelope polyprotein, accession number GI:331936), which removes a putative Golgi retention signal of RVFV G, did not significantly increase RVFV G content on cell surfaces as demonstrated by immunofluorescence studies and did not result in increased chimVLP yields (data not shown).

Optimization of VLP production is important for the ability to scale-up for the generation of material required for non-human primate and livestock studies and ultimately clinical grade vaccine production. First, as seen in Fig. 2A, the ratio of transfected expression plasmid for the RVFV G and N proteins influences VLP yields. Second, optimal generation of VLPs is clearly observed when the N plasmid is included, and increasing amounts of N expression plasmid enhances the generation of RVFV VLPs. Similar findings were also reported for the generation of UUK VLPs (Overby et al., 2006). The addition of a RVFV-specific minigenome did not significantly increase the N content of generated VLPs despite the fact that VLPs were able to package minigenome and transfer reporter gene activity into VLP-infected cells (M. Bouloy and R. Flick, unpublished data). This contradicts previous findings by Overby et al. (2006) who showed that omission of a minigenome leads to almost no nucleoprotein incorporation into UUK VLPs. Generation of VLPs is optimal between 24 and 60 h post-transfection (Fig. 2B) as determined via time course experiments. Furthermore, multiple harvests during a 120-h time period post-transfection results in higher VLP yields compared to less frequent harvests. This is consistent with our previous studies which show that multiple harvests of Ebolavirus and Lassa virus chimVLPs yields substantially more VLPs compared to a single harvest or collections at intervals longer than 12 h (data not shown). This might reflect VLP stability or binding to the producing cells.

While inactivated viral vaccines are often ineffective at eliciting neutralizing antibodies (Green et al., 2001), robust vaccines can elicit the development of neutralizing antibodies that are maintained for prolonged times (Kan et al., 2007). A PRNT50 of 1:40 is generally accepted as protective against RVF disease in mice (Peters et al., 1986), rats (Anderson et al., 1991a; Anderson, Slone, and Peters, 1987), hamsters (Niklasson et al., 1984) and Rhesus macaques (Peters et al., 1988). Pittman et al. (1999) examined the neutralizing antibody responses in 598 human subjects vaccinated with the TSI-GSD-200 inactivated RVFV vaccine. 540 (90.3%) had serum neutralizing antibody titers >1:40 after their primary series of three injections while 58 individuals (9.7%) had titers of <1:40. PRNT50 >1:40 was maintained for 183 days in 85% of recipients but decreased to only 35% at 1 year indicating that durability might be an issue with this particular vaccine.

Correlation between the development of neutralizing antibodies and protective efficacy for RVFV vaccines has also been shown more recently in several systems. Naslund et al. (2009) showed that five of six mice vaccinated with 1 x 10^6 RVF VLPs developed neutralizing antibody titers (PRNT50) from 250 to 1250. Eleven of 12 vaccinated mice were protected from lethal challenge. Bird et al. (2008) showed in a rat model that neutralizing antibody titers (PRNT50) of 1:640–1:7040 were obtained with a highly attenuated RVFV strain lacking the NSs and NSm genes that is 100% protective from lethal challenge. Here we demonstrate that RVF VLPs induce neutralizing antibody titers of ≥1:640 detectable 6 months post-immunization (Fig. 3), indicating the robustness and durability of the VLP-based RVFV vaccine candidates. Interestingly, as seen in Fig. 3, while similar neutralizing antibody titers are generated by RVF VLPs with or without N, these results do not correlate with vaccine efficacy in the mouse model, as seen in Fig. 5, where N-containing RVF VLPs are substantially more efficacious compared to VLPs lacking RVFV N. As demonstrated with DNA vaccines expressing only the RVFV M ORF, while it is likely that immunity to RVFV is determined by the response to the RVFV G (Lagerqvist et al., 2009; Spik et al., 2006; Wallace et al., 2006), the results described above suggest that N might represent an important component of an efficacious RVFV vaccine. It has been demonstrated that both RVFV N (Lagerqvist et al., 2009; Wallace et al., 2006) and Toscana virus (a related bunyavirus) N (Gori Savellini et al., 2006) are partly protective against lethal challenge in mice. Interestingly, while previous passive transfer studies would suggest that the generation of neutralizing antibodies to RVFV Gn and Gc is
predictive of vaccine efficacy (Besselaar and Blackburn, 1991; Schmaljohn et al., 1989), our RVF VLP without N, while sufficient for the generation of neutralizing antibodies (see Fig. 3), is not efficacious in the mouse challenge model (see Fig. 5).

Furthermore, we were able to demonstrate that RVF VLPs clearly induce antigen-specific cytokine secretion by isolated splenocytes from vaccinated animals (Fig. 4). Consistent with these results, Ebolavirus VLPs produced in mammalian and insect cells have been shown to stimulate secretion of cytokines such as IL-6, IL-10, IL-12, and TNF-α from dendritic cells (Bosio et al., 2004; Ye et al., 2006). Taken together, measurement of immune correlates clearly demonstrate that VLPs are immunogenic; however, only vaccine efficacy studies can clearly demonstrate the potency of vaccine candidates.

We therefore employed two different rodent models to determine RVF VLP-based vaccine efficacy. The results shown in Figs. 5 and 6 clearly demonstrate that our RVF VLP vaccine candidates are partially protective in mice and fully protective in rats. Importantly, RVFV ZH501 challenge in rats was performed with a 2 log10 higher dose than is often reported in the literature (see Bird et al., 2008) to ensure 100% lethality in the model and to demonstrate vaccine potency. Overall, this novel approach of using chimeric RVF VLPs as vaccine candidates yielded promising immunological and efficacy data in two different rodent models and sets a strong precedent for the generation of an efficacious vaccine against RVFV that is urgently needed for the high containment laboratory worker, indigenous people in endemic areas, and for the Strategic National Stockpile and National Veterinary Stockpile as protection against the emergence of this disease and for a potential bioterror event.

Materials and methods

RVFV glycoprotein (G) expression plasmids

RNA was purified from MP12-infected Vero cells using a QIAamp Viral RNA Mini Kit (Qiagen). MP12 sequons were chosen as the source of the glycoprotein gene because it allowed us to perform initial challenge studies with MP12 under lower containment conditions (data not shown). cDNA was prepared using RT PCR (Thermoscript RT-PCR Kit) with oligo dT primers. A 3.3 kb 5′-truncated fragment of the RVFV M segment was amplified from cDNA using Phusion HF polymerase (Phusion High Fidelity PCR Mastermix, Finnzymes) and RVFV M segment-specific primers: 5′-GCAATCGATGCGAGGATGCAATGACAGTC and 5′-GCACTGAGCCTAGGCGCCCTTTAAGTGGCACG. This results in translation of the RVF glycoprotein precursor from the 4th available start codon to facilitate optimal glycoprotein expression (Collett et al., 1985; Gerrard and Nichol, 2007; Suzich et al., 1990). Next, a Kozak sequence (Kozak, 1984a; Kozak, 1984b; Kozak, 1987; Kozak, 2002) was added immediately 5′ to the ATG start codon by PCR amplification using Phusion HF polymerase with the following RVFV M segment specific oligos: 5′-TTAATAATGAATTCGCCACCATGGCAGGGATTGCTGAGC. This generates a product with a Kozak sequence (Kozak, 1984a; Kozak, 1984b; Kozak, 1987; Kozak, 2002) immediately 5′ to the start codon and 5′ and 3′ KpnI and XhoI restriction sites, respectively. This product was cloned into pCAGGS expression vector via KpnI/XhoI restriction endonuclease sites. Plasmid DNA used for transfections was prepared (0.323 mg/ml, NucleoBond column-purified (Machery-Nagel) by the Nature Technology Corporation (Lincoln, NE, USA).

RVFV N expression plasmid

RVFV N cDNA was amplified from MP12 RNA by RT-PCR (ThermoScript RT-PCR Kit) with primers specific for the S segment: 5′-ATTATGTGATCCCGCCACGATGACATATCAAGATCGCTTGACAT and 5′-ATTATTCCAGATTTAGGCTGCTTCTTTTGAACCTTGAG. This generates a product with a Kozak sequence (Kozak, 1984a; Kozak, 1984b; Kozak, 1987; Kozak, 2002) immediately 5′ to the start codon and 5′ and 3′ KpnI and XhoI restriction sites, respectively. This product was cloned into pCAGGS expression vector via KpnI/XhoI restriction endonuclease sites. Plasmid DNA used for transfections was prepared (3.024 mg/ml, CsCl-purified) by the Recombinant DNA Laboratory, Sealy Center for Molecular Medicine, UTMB (Galveston, TX, USA).

RVF VLP preparation

293-gag cells (HEK-293 cells (CRL-1573, ATCC) that constitutively express MoMLV gag and pol) were cultured in poly-d-lysine coated 150 mm tissue culture dishes (Falcon) at 1.2 × 106 cells per plate in DMEM (Gibco) with 10% FBS (Hyclone) with 1% penicillin/streptomycin (pen/strep, 1000 U/ml and 1000 μg/ml, Gibco), and 2 mM l-glutamine (Gibco) at 37°C, 5% CO2 overnight. Media was removed and transfection of RVFV G and N expression plasmids was performed using Lipofectamine 2000 reagent (Invitrogen) with Opti-MEM I media (Gibco). Transfection media was removed at 4 h post-transfection and replaced with 30 ml DMEM media plus 10% FBS, 1% pen/strep and 1% l-glutamine. Supernatants were harvested at 24, 36, 48, 72, 96 and 120 h post-transfection, and each time the cells were cultured with 30 ml of fresh medium. Supernatants were pooled and clarified by centrifugation at 2700 × g at 4°C for 10 min. Samples were concentrated to 150 ml via tangential flow filtration through Pellicon® 2 “Mini” Filter (0.1 μm Biomax® 300K polyethersulfone, screen type C, Millipore). Purification of RVF chimVLPs was performed by centrifugation of concentrated VLP preparations through a 20% sucrose cushion in PBS using Beckman Ultraclear ultracentrifuge tubes in a SW28 rotor at 26,000 rpm at 4°C for 2 h with a Beckman L-80 ultracentrifuge. Samples were then resuspended in 5 ml of sterile 0.9% NaCl (Baxter). RVF VLPs from 293 cells were prepared using the same methodology.

Western blot analysis

RVFV chimVLPs were combined with 4 × LDS buffer (Invitrogen) and 50 mM Dithiothreitol (DTT, Sigma), heated to 95°C for 10 min, then fractionated by NuPAGE 4-12% Bis-Tris Gels (Invitrogen) for protein size comparison a pre-stained protein molecular weight marker (SeeBlue Plus 2, Invitrogen) was used. Proteins were then transferred to methanol-activated PVDF membrane (Invitrogen) which was subsequently incubated 16 h in 1% nonfat dry milk in PBS. Membranes were washed 3 × for 10 min in 0.05% Tween20 in PBS and probed with primary antibodies for 1 h at room temperature (RT): monoclonal RVFV GN antibodies at 1:8,000 (ProSci Inc., Poway, CA, USA, 4F8C8, developed against the GN-specific peptide AEDPHLRNRPGKGH), monoclonal RVFV GN antibodies 1:5,000 (ProSci Inc., 14G1B11, developed against the GC-specific peptide QTRNDKTFAASKGN), RVFV N ascites 1:2,000 (kindly provided by Dr. Robert B. Tesh, University of Texas Medical Branch, USA) and rabbit polyclonal MoMLV gag antibodies 1:5,000 (kindly provided by Dr. Chinglai Wang, Emory University, USA) for 1 h at RT. Membranes were washed 3 × as above, then incubated with either AP-conjugated goat anti-mouse antibodies at 1:5,000 (for GN, GC, and N; Jackson ImmunoResearch, West Grove, PA, USA) or AP-conjugated rabbit anti-goat (for MoMLV gag; Southern Biotech, Birmingham, Alabama, USA).
USA). Membranes were then washed 3 × as described above. Protein bands were visualized using 1-Step NBT/BCIP solution (Pierce). For quantitative analysis, Western blots were analyzed using ImageJ software (Burger and Burge, 2008). Image colors were inverted and background subtracted. The average of three integrated density readings per band was determined. Maximum value was set to equal 100% and the remaining values were converted to percentages relative to the highest reading.

Transmission electron microscopy (TEM)

TEM was performed at the University of Iowa Central Microscopy Research Facility (University of Iowa, USA). 293 cells were fixed 12 h with glutaraldehyde (Acros Organics, Geel, Belgium; final concentration of 2.5%) 60 h post-transfection of RVFV G and N expression plasmids. Cells were washed with PBS pH 7.2 and then 3 × with 0.1 M sodium cacodylate buffer. Cells were then fixed 1 h with 1% osmium tetroxide and washed 3 × with 0.1 M sodium cacodylate buffer. Subsequently, cells were rinsed in distilled water for 1 min and then treated with 2.5% uranyl acetate in distilled water for 20 min. Cells were then equilibrated into ethanol in three 15-min steps (50%, 75% and 95%) and then equilibrated in 2:1 ethanol/epon resin (Epon 12, Ted Pella) and then 1:2 ethanol/epon for 1 h and finally 100% epon for 2 h. Samples were then placed in fresh epon at 65 °C for 12 h. Blocks were then subjected to microtomy to generate 70-nm thin sections. SEM was performed at the University of Iowa Central Microscopy Research Facility (University of Iowa, USA). 293 cells were maintained at 37°C, 5% CO2, and 90% humidity. Supernatants were harvested for analysis at 24, 96, and 168 h post-stimulation. Cytokines in cell culture supernatant were analyzed by a bead-based multiplex system (Bioplex, BioRad) according to manufacturer’s instructions. Secretion of the following was assessed: IL-2, IL-4, IL-5, and IFN-γ.

Mouse challenge experiments

BALB/c, H2d haplotype, α13 galactosyltransferase-KO transgenic mice (Thall et al., 1995), kindly provided by NewLink Genetics Corporation, were immunized on days 0, 9, and 20 with RVFV chimVLPs. Spleens were harvested from three mice and one PBS-vaccinated control mouse (31 days post final vaccination) and each placed in separate petri dishes with sterile Gey’s Balanced Salt Solution (Sigma). Each spleen was slightly minced and pressed through a Collector™ Tissue Sieve (Belco). Cells were filtered through a 40 μm filter (BD Falcon 40 μm strainer) and centrifuged at 800 × g for 10 min. Cells were then resuspended in Gey’s solution and centrifugation through underlayered Lympholyte-M (Cedarlane Labs, Burlington, NC, USA) at 1500 × g for 20 min (with no brake). Splenocytes were harvested from the interface and washed 3 × in Gey’s solution. Viable cells were plated to 24-well dishes (Corning, Corning, NY, USA) at 8 × 10⁶ cells/mL. 1 × 10⁷ TCID₅₀/ml heat-inactivated flu virus (mouse-adapted A/HK/1/68 strain; Abdel-Motal et al., 2007) or 5.6 × 10⁵ pfu/ml heat-inactivated RVFV MP-12 (Caplen, Peters, and Bishop, 1985) was added to test wells. Cells were maintained at 37°C, 5% CO₂, and 90% humidity. Supernatants were harvested for analysis at 24, 96, and 168 h post-stimulation. Cytokines in cell culture supernatant were analyzed by a bead-based multiplex system (Bioplex, BioRad) according to manufacturer’s instructions. Secretion of the following was assessed: IL-2, IL-4, IL-5, and IFN-γ.

Rat challenge experiments

Eight-week-old female Wistar–Furth rats (Harlan Laboratories, Indianapolis, IN) were immunized 3 × at 2-week intervals i.p. with 1 ml of RVF chimVLP vaccine candidate or 1 ml sterile saline (Baxter, Deerfield, IL) combined with 250 μl of Sigma Adjuvant System (prepared according to manufacturer’s recommendations). Rats were
transferred to the Robert E. Shope BSL-4 facility at the University of Texas Medical Branch at Galveston for subcutaneous (s.c.) challenge with 10^3 pfu of RVFV ZH501 60 days post final booster. Rats were monitored daily for weight change and signs of disease. All animal experiments were approved by Iowa State University and the University of Texas Medical Branch IACUC committees.

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