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SARS vaccine based on a replication-defective recombinant vesicular stomatitis virus is more potent than one based on a replication-competent vector

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ABSTRACT

A SARS vaccine based on a live-attenuated vesicular stomatitis virus (VSV) recombinant expressing the SARS-CoV S protein provides long-term protection of immunized mice from SARS-CoV infection (Kapadia, S.U., Rose, J. K., Lamirande, E., Vogel, L., Subbarao, K., Roberts, A., 2005. Long-term protection from SARS coronavirus infection conferred by a single immunization with an attenuated VSV-based vaccine. Virology 340(2), 174–82.). Because it is difficult to obtain regulatory approval of vaccine based on live viruses, we constructed a replication-defective single-cycle VSV vector in which we replaced the VSV glycoprotein (G) gene with the SARS-CoV S gene. The virus was only able to infect cells when pseudotyped with the VSV G protein. We measured the effectiveness of immunization with the single-cycle vaccine in mice. We found that the vaccine given intramuscularly induced a neutralizing antibody response to SARS-CoV that was approximately ten-fold greater than that required for the protection from SARS-CoV infection, and significantly greater than that generated by the replication-competent vector expressing SARS-CoV S protein given by the same route. Our results, along with earlier studies showing potent induction of T-cell responses by single-cycle vectors, indicate that these vectors are excellent alternatives to live-attenuated VSV.

Introduction

SARS (severe acute respiratory syndrome) emerged in the fall of 2002 in China but soon caught the world's attention as it quickly spread to 28 countries. By the end of 2003 the World Health Organization reported over 8000 probable cases of SARS, a fifth of which occurred in health care workers. The overall fatality rate was 9.6%, but in people over the age of 60, the rate exceeded 50%. (http://www.who.int/csr/sars/en/WHOconsensus.pdf; Peiris et al., 2004)

The etiological agent was quickly identified as a coronavirus (CoV) (Drosten et al., 2003; Ksiazek et al., 2003), and the 30 kb genome sequence revealed a common coronavirus genome organization (Marra et al., 2003; Rota et al., 2003). Six major open reading frames were identified. Of those, four encoded the major structural proteins: spike (S), membrane (M), nucleocapsid (N) and envelope (E). M, N and E are involved in viral assembly and budding. S, the major glycoprotein, binds the cellular receptor, ACE 2 (Li et al., 2003), and mediates entry by a class I viral fusion mechanism (Bosch et al., 2003).

There have been no reported cases of SARS since 2004; however sources of the SARS-CoV still exist. Animal carriers of the virus including Himalayan palm civets, raccoon dogs and bats have been identified (Guan et al., 2003; Lau et al., 2005; Li et al., 2005). Several cases of laboratory-acquired SARS have also been reported. Because SARS-CoV has not been eradicated, there is still a potential for human infections. A SARS vaccine may be important in controlling future outbreaks.

Several experimental vaccines have been constructed and tested. These include DNA vaccines, protein subunit vaccines, inactivated SARS-CoV vaccine and recombinant viral vaccines (Gillim-Ross and Subbarao, 2006). The SARS-CoV S glycoprotein has been used as the antigen in the development of most of these SARS vaccines because it is the target of virus neutralizing antibody. We previously reported the development of an experimental VSV-based SARS vaccine. VSV (vesicular stomatitis virus) is a negative strand RNA virus that belongs to virus family *Rhabdoviridae* (Kapadia et al., 2005). Attenuated vectors derived from VSV have been used extensively as experimental vaccine candidates (Daddario-DiCaprio et al., 2006a,b; Egan et al., 2004; Geisbert et al., 2005; Jones et al., 2005; Kahn et al., 2001; Natuk et al., 2006; Palin et al., 2007; Ramsburg et al., 2004; Reuter et al., 2002; Roberts et al., 1999, 1998, 2004; Rose et al., 2001; Schlereth et al., 2003, 2000). They induce strong antibody and cellular immune responses, and with the exception of some rural populations in Central and South America, there is negligible seropositivity to VSV in the human population (Reif et al., 1987) making them attractive candidates for human vaccination. For populations with pre-existing immunity to VSV, non-endemic VSV serotype vectors can be used. VSV also grows to high titers in cell lines approved for vaccine production.

In our initial study (Kapadia et al., 2005) we showed that a VSV recombinant expressing the SARS-CoV S protein was capable of

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generating neutralizing antibodies against SARS-CoV in mice. Furthermore, the immunized mice were protected from a SARS-CoV challenge. We also showed that a humoral response was sufficient for protection. In the current study we generated and tested the effectiveness of a VSV recombinant that is capable of undergoing only one round of infection because it lacks the gene encoding the VSV glycoprotein (G). Use of such a replication-deficient vector would overcome the complex regulatory issues related to approval of live-virus vectors for use in humans. However, production of such vectors would require a qualification of a cell-line that expresses VSV G or some plasmid DNA based complementation. Furthermore, a single-cycle viral vaccine would alleviate concerns over potential risks related to the use of live viral vectors in individuals with weakened immune systems. In order to evaluate this vector as a SARS vaccine candidate, we also developed a SARS-CoV neutralization assay using a pseudotyped VSV recombinant expressing a green fluorescent protein.

Results

Construction and characterization of a single-cycle VSV encoding the SARS-CoV S protein

In order to recover a single-cycle VSV recombinant encoding the S protein of SARS-CoV, the VSV glycoprotein (G) gene in a plasmid expressing the VSV anti-genome was replaced with a gene encoding SARS-CoV S (Fig. 1A). The resulting plasmid, pVSVΔG–SARS S, was used to recover a virus, VSVΔG-S on BHK-21 cells expressing VSV G. Because G is required for virus entry, VSV recombinants lacking the G gene must be complemented with G in order to produce infectious particles. Viruses

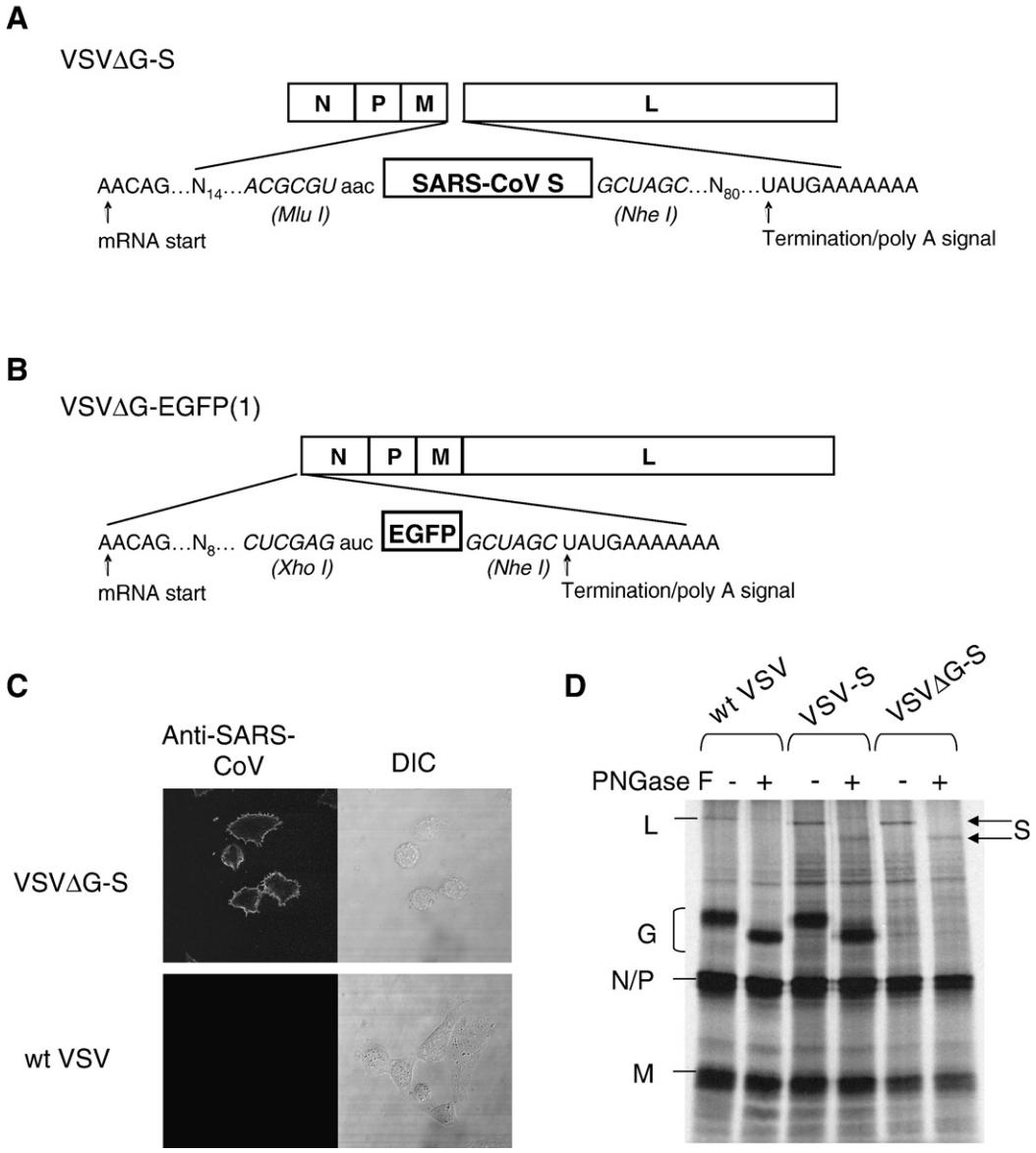

Fig. 1. SARS-CoV S expressed by a single-cycle VSV recombinant. (A) The VSV G gene is replaced by the SARS-CoV S gene in the VSV genome to yield the VSVΔG-S genome. The RNA sequences are shown in the (+) anti-genomic sense. (B) The VSVΔG-EGFP1 lacks the VSV G gene and has an EGFP gene inserted into the first position of the genome. The RNA sequences are shown in the (+) anti-genomic sense. (C) BHK-21 cells were infected with either VSVΔG-S or wt VSV. Cells were fixed, and SARS-CoV S was visualized by indirect immunofluorescence. The fluorescence images are shown on the left, and differential interference contrast (DIC) images are shown on the right. (D) Lysates of metabolically labeled BHK-21 cells infected with wt VSV (lanes 1 and 2), VSV-S (lanes 3 and 4) or VSVΔG-S (lanes 5 and 6) were analyzed by SDS-PAGE. The lysates were also treated with PNGase F to remove N-linked glycans from proteins (lanes 2, 4, and 6).

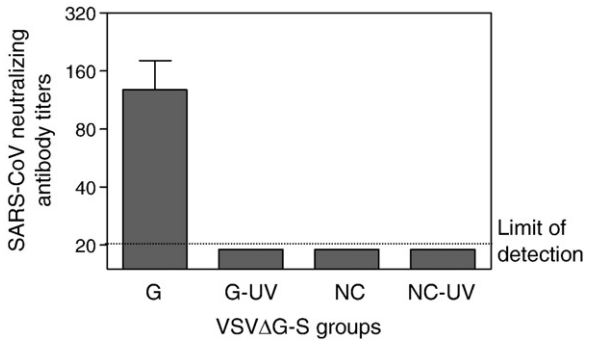

Fig. 2. VSV G is needed to induce a SARS-CoV neutralizing response. Mice were immunized i.m. with VSVΔG-S either G-complemented or (G) or non-complemented with any viral glycoproteins (NC). Each virus was administered live or UV-inactivated (UV). Serum SARS-CoV neutralizing antibody titers one month post-immunization were determined in each group and used as a read out for replication *in vivo*. Error bars indicate standard error of the mean. (N=5).

complemented with G can infect cells for a single cycle, but do not propagate further in the absence of a complementing G protein. When VSVΔG-S was used to infect BHK-21 cells, we observed only single infected cells and no virus spread, consistent with the absence of an encoded VSV G protein.

To determine if the S protein was expressed by this recombinant, we examined cells using indirect immunofluorescence microscopy. BHK-21 cells were infected with VSVΔG-S or wild type (wt) VSV, fixed, and then incubated with serum from a SARS-CoV-infected mouse. A secondary, Alexa Fluor 488-conjugated anti-mouse antibody was used for visualization by confocal microscopy (Fig. 1C). We found that the SARS-CoV S protein was expressed on the cell surface as indicated by the strong surface fluorescence signal visible in cells infected with VSVΔG-S but not on control cells infected with wt VSV.

To evaluate viral protein expression further, we infected BHK-21 cells with wt VSV, VSV-S (Kapadia et al., 2005) or VSVΔG-S and metabolically labeled cells with [^{35}S]-methionine. Lysates of radiolabeled cells were analyzed by SDS-PAGE. Because VSV infection shuts off host protein synthesis, the five VSV proteins L, G, N, P and M are readily seen without immunoprecipitation (Fig. 1D, lane 1). VSV-S-infected cells expressed the SARS-CoV S protein in addition to the five VSV proteins (Fig. 1D, lane 3). VSVΔG-S-infected cells expressed S and all of the VSV proteins except G (Fig. 1D, lane 5). Because S is a highly glycosylated protein, we also treated the lysates with PNGase F to remove glycans in order to further characterize S. After digestion, S migrated faster on the gel (Fig. 1D, lanes 4 and 6), a change consistent with removal of the predicted 17 glycans (Kapadia et al., 2005).

VSVΔG-S is a single-cycle vector in tissue culture and in mice

Because a low level of S protein of SARS-CoV is incorporated into VSV particles (data not shown), it is possible that the S protein might mediate infection in the absence of G. To determine if this S protein could mediate infection of VSVΔG-S, we infected Vero E6 cells [cells that express the SARS-CoV receptor, ACE2 (Ksiazek et al., 2003; Li et al., 2003)] with G-pseudotyped VSVΔG-S. Using an immunofluorescence microscopy assay for observing VSV N protein expression, we saw single infected cells after 6 h but observed no infected cells after 24 h despite a near confluent monolayer of live Vero E6 cells. This result indicates that VSVΔG-S is not capable of a second round of infection. Additionally, we passaged VSVΔG-S through BHK-21 cells to yield progeny lacking VSV G and attempted to infect Vero E6 cells with this virus stock. Even though we could detect S protein in these non-complemented virions by Western blot, we did not observe any infection of Vero E6 cells with these particles. These results indicated that the S protein present on the virion was not capable of mediating VSV entry in tissue culture. This is consistent with reported results showing that the full-length S protein, as is encoded in VSVΔG-S, is not capable of mediating infection of pseudotyped VSV (Fukushi et al., 2005).

To determine if VSVΔG-S is able to replicate *in vivo* without VSV G, we inoculated mice intramuscularly (i.m.) with non-complemented VSVΔG-S. If there were any infection by this virus, we anticipated that there might be detectable immune responses to the S protein. We measured SARS-CoV neutralizing antibody response as a measure of replication. To control for possible immune responses to S protein on the surface of particles in the inoculum, we also administered UV-inactivated, non-complemented VSVΔG-S. As additional controls we immunized two groups of mice with either G-complemented VSVΔG-S or UV-inactivated, G-complemented VSVΔG-S. A dose of 5×10^5 pfu (plaque forming units) of the G-complemented VSVΔG-S was used. An equivalent particle dose of the non-complemented virus was assessed from the amount of N protein in the virus preparation as determined by Western blot.

One month after inoculation, serum was collected from each animal and the SARS-CoV neutralization titers were determined. Only the G-complemented VSVΔG-S-inoculated animals generated any measurable neutralizing titers to SARS-CoV (Fig. 2). They averaged 1:128. The animals in the remaining groups including those inoculated with non-complemented VSVΔG-S made no measurable neutralizing antibody response (even at an antibody dilution of 1:5) indicating that significant replication was not occurring. UV-inactivated G-complemented VSVΔG-S did not induce a neutralizing antibody response, indicating that one round of replication is essential for a response.

We also assessed the immune responses to the VSV vector in these animals. We used the serum from each animal to stain VSV-infected cells and observed VSV N expression by immunofluorescence microscopy. All animals immunized with live G-complemented VSVΔG-S had an antibody response to N, while animals inoculated with non-complemented VSVΔG-S had no detectable response to VSV. This further supports the idea that VSVΔG-S is not infectious in animals without VSV G.

Immune responses to VSVΔG-S

In order to test the potential of our single-cycle VSV recombinant as a SARS vaccine, we conducted a study including five groups of mice. The first group included three control mice that received wt VSV intranasally (i.n.). The second group of three mice was inoculated with wt VSV i.m. The third group of six mice was immunized with VSV-S administered i.n., while the fourth group of six mice received VSV-S i.m. The last group of five mice was vaccinated with VSVΔG-S i.m. A single vaccine dose of 5×10^5 pfu was administered. Serum was collected from all mice at 5, 9 and 13 weeks post-immunization.

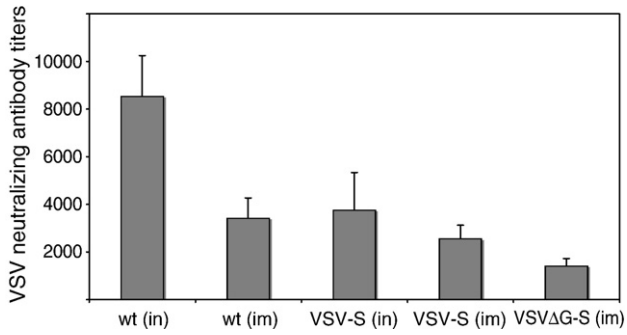

Fig. 3. VSV neutralizing antibody response. The VSV neutralizing antibodies titers of samples collected at 5 weeks post-immunization are shown. The mean reciprocal dilution giving 100% neutralization is shown for each group. Error bars indicate standard error of the mean.

To verify that all mice had been infected with the vectors, we measured VSV neutralizing antibody titers in the serum of individual mice at five weeks post-infection (Fig. 3). VSV G protein is the target of VSV neutralizing antibodies (Kelley et al., 1972). All mice made measurable neutralizing antibody titers to VSV consistent with successful infection. Wt VSV administered i.n. produced the highest VSV neutralizing titers (mean titer of 1:8533) consistent with previous results (Kapadia et al., 2005). Wt VSV given i.m. and VSV-S given either i.m. or i.n. produced mean VSV neutralizing titers between 1:2560 and 1:3760. The VSVΔG-S group produced a lower VSV neutralizing titer (mean of 1:1408) consistent with the fact that this virus does not encode a VSV G protein but does carry G protein on the particles generated by complementation with VSV G. These results indicate that all mice had been effectively inoculated.

Development of a novel assay for SARS-CoV neutralizing antibody

Because a humoral response to the SARS-CoV S protein is sufficient for protection against SARS-CoV infection, we wanted to determine the SARS-CoV neutralizing antibody titers in the serum of mice in this study. In a previous study, we had used direct neutralization of SARS-CoV to determine SARS-CoV neutralizing titers. In order to circumvent the level of bio-containment required for this assay, we developed and validated an assay using a VSVΔG virus expressing EGFP and complemented with SARS-CoV S protein, the target of SARS-CoV neutralizing antibodies.

We first generated a VSV recombinant, VSVΔG–EGFP1. The genome of this virus (Fig. 1B) has four VSV genes, N, P, L and M, and an EGFP gene in the first position of the VSV genome to promote maximal EGFP expression. Next we inserted the gene for a tagged SARS-CoV S protein with its cytoplasmic tail replaced with an HA epitope tag (SΔtail-HA) into a mammalian expression vector, pCAGGS (Niwa et al., 1991). The deletion of the tail is required for infection in the context of pseudotyped viruses (Fukushi et al., 2005; Giroglou et al., 2004; Moore et al., 2004). This plasmid was transfected into BHK-21 cells. When the transfected cells were expressing SΔtail-HA protein, they were infected with VSVΔG–EGFP1 complemented with VSV G. The virus was adsorbed for 1 h, and the cells were then washed three times with PBS in order to remove the input particles. The media was replaced and the infection was allowed to continue for 24 h. The resulting pseudotyped virus, VSVΔG–EGFP1/SΔtail-HA, was present in the media collected from these cells.

We next determined if the pseudotyped VSVΔG–EGFP1/SΔtail-HA could be used to assay for SARS-CoV neutralizing antibodies. We incubated the pseudotyped virus with antiserum from mice inoculated with either wt VSV (which have neutralizing antibody directed to VSV G only), VSV-S (which have neutralizing antibody to VSV and SARS-CoV), or SARS-CoV (which have antibody to SARS-CoV only) at a dilution of 1:50 to ascertain which antibodies were capable of neutralizing the pseudotyped virus. We used VSVΔG–EGFP1 pseudotyped with VSV G as a control to measure neutralizing antibodies that react with VSV G. Following a one-hour incubation at 37 °C, the virus-serum mixtures were then transferred to a monolayer of Vero E6 cells, which express the SARS-CoV receptor, ACE 2 (Ksiazek et al., 2003; Li et al., 2003). The cells were incubated at 37 °C for 18 h and then fixed with 3% paraformaldehyde. We determined infection by observing EGFP expression using fluorescence microscopy.

Infection of Vero E6 cells by the VSVΔG-EGFP1/SΔtail-HA pseudotypes was not neutralized by antibodies to VSV, but was neutralized by antibodies to VSV-S (which contains antibodies to VSV and S) or SARS-CoV (which contains antibodies to S). In contrast VSVΔG-EGFP1/G was not neutralized by antibody to SARS-CoV, but was neutralized by antiserum to VSV or VSV-S (Fig. 4). These results show that neutralization of the S-pseudotyped virus was specific for antibody to SARS-CoV S.

The VSVΔG–EGFP1/SΔtail-HA pseudotype assay is as sensitive as the direct SARS-CoV neutralization assay

We next compared the sensitivity of our neutralization assay with the standard assay using serum standards assayed previously with the direct SARS-CoV neutralization assay. We used sera from mice

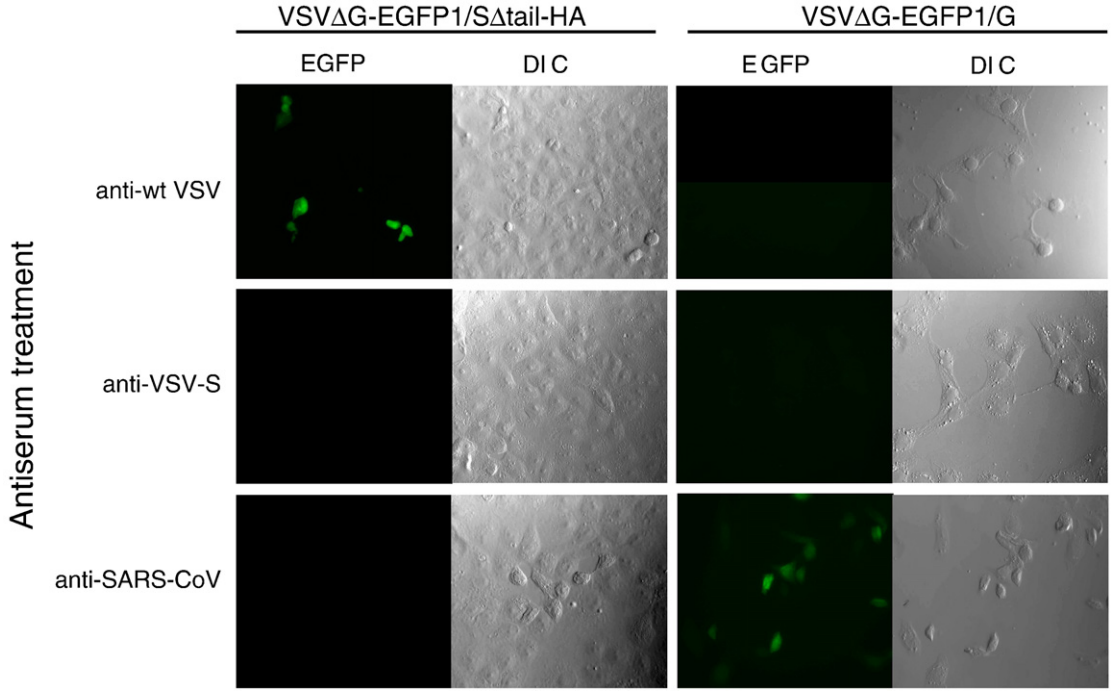

Fig. 4. Specific neutralization of VSVΔG-EGFP1/SΔtail-HA by anti-S antibody. VSVΔG-EGFP1 pseudotyped with either SΔtail-HA or VSV G proteins were incubated with antiserum from mice immunized with wt VSV, VSV-S or SARS-CoV as indicated. The pseudotypes were then transferred to Vero E6 cells. Infection was determined by EGFP expression. Both fluorescence images and differential interference contrast (DIC) images are shown for each field.

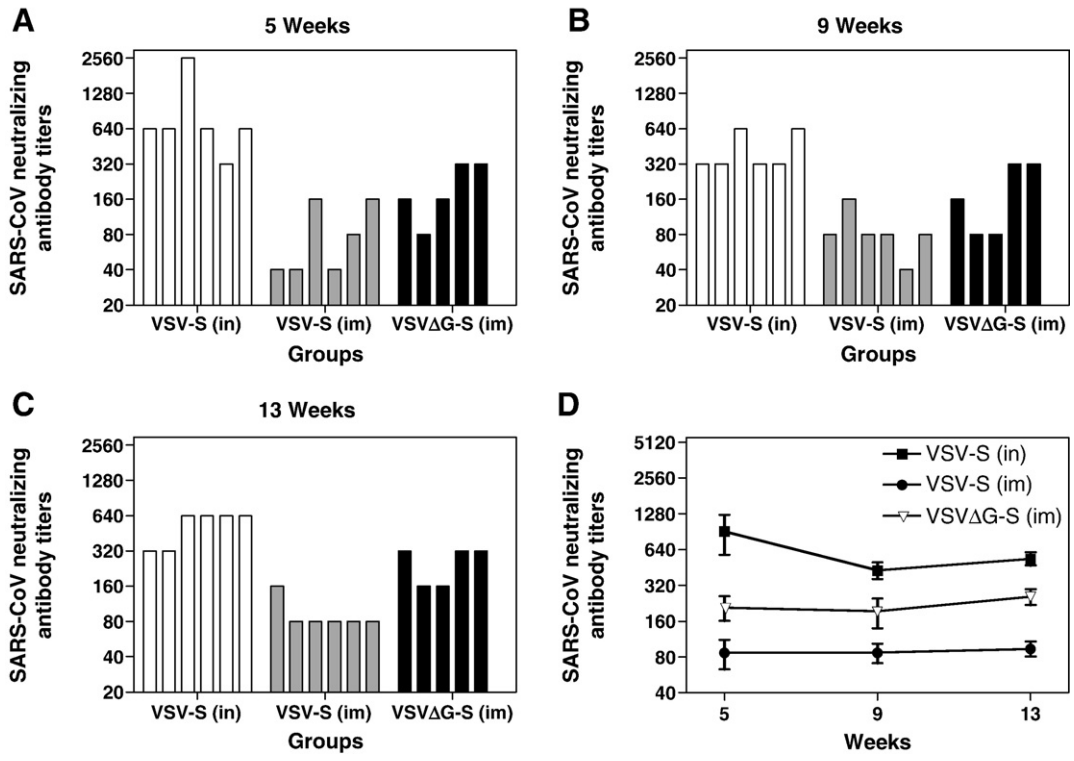

Fig. 5. SARS-CoV neutralizing antibody responses to each vector. The SARS-CoV neutralizing antibody titers of serum of individual mice at 5 weeks (A), 9 weeks (B) and 13 weeks (C) post-immunization are given. The reciprocal dilutions giving 100% neutralization for each group are shown. The mean reciprocal dilutions giving 100% neutralization for each group over time are summarized (D). Mice immunized with wt VSV, either i.m or i.n., had no detectable SARS-CoV neutralizing antibodies. Error bars indicate standard error of the mean.

immunized with either wt VSV, VSV-S or SARS-CoV from our previous SARS vaccine study (Kapadia et al., 2005). SARS-CoV neutralizing antibody titers of these sera were determined by incubating VSVΔG-EGFP/SΔtail-HA virus with serial dilutions of these sera, and the virus-serum mixtures were transferred to a monolayer of Vero E6 cells. Infection was determined by observing EGFP expression by fluorescence microscopy 18 h after infection. The titer was defined as the highest dilution that completely neutralized VSVΔG-EGFP1/SΔtail-HA. There was no detectable neutralizing activity in serum from mice vaccinated with wt VSV. The titers in serum samples from VSV-S- and SARS-CoV-inoculated mice were determined to be 1:40 and 1:20 respectively in pseudotype assay. These titers of these sera were 1:32 and 1:12 in the direct assay. Furthermore these sera were from mice that were able to control SARS-CoV infection upon challenge. Since an antibody response is sufficient for protecting against SARS-CoV (Bisht et al., 2004; Kapadia et al., 2005; Yang et al., 2004), a titer neutralizing titer of as low as 1:20 is indicative of protection.

VSVΔG-S induces a response indicative of protection

We then used this neutralization assay to measure the neutralizing antibody titers in the serum of the mice in our current study (Fig. 5). No SARS-CoV-neutralizing antibodies were detected in animals that were infected by wt VSV. There was little variability between individual mice within a group at the three time points measured (Fig. 5A, B and C). Notably animals made neutralizing antibodies titers that were considerably greater than 1:20, a titer we determined previously to be protective against SARS-CoV challenge. Animals infected by VSV-S i.n. produced the most robust response with a mean titer of 1:906 five weeks post-vaccination (Fig. 5D). This level dropped by to 1:426 by nine weeks post-vaccination. The group immunized with VSVΔG-S also made a strong antibody response with average titers approximately 1:200 at all time points tested. This response was about two-fold greater than that seen in the group immunized i.m. with the replication-competent VSV-S. This difference was statistically significant at 13 weeks post-immunization ($p=0.0087$, Mann–Whitney test). A similar trend was previously reported with a single-cycle VSV vector expressing the HIV Env protein. It generally generated a better T-cell response to HIV Env than the replication-competent VSV vector expressing HIV Env when administered i.m., though the difference was not statistically significant (Publicover et al., 2005).

Discussion

Regulatory approval for the use of replication-competent VSV-based vaccine vectors in humans has been slow because of concerns about potential pathogenesis. We therefore have developed single-cycle VSV-based vectors lacking the VSV G gene that can infect cells, but cannot produce infectious particles (Schnell et al., 1997). We report here that such a replication-defective vector expressing the SARS-CoV S protein is highly effective at generating SARS-CoV neutralizing antibody in animals when given i.m. and is even better than a replication-competent VSV vector expressing SARS-CoV S given by the same route.

We were concerned that the single-cycle VSVΔG-S vaccine vector described here might be able to mediate multiple rounds of infection because some S protein is incorporated into virions. However, we did not detect any infection by non-G-complemented VSVΔG-S particles in cells expressing the SARS-CoV receptor. Furthermore, when we inoculated mice with these non-complemented pseudotyped particles, we saw no immune responses to S or to VSV N indicating that no significant infection occurred. Others have also reported that full-length SARS-CoV S was not able to mediate entry of VSV and found that a deletion in the carboxy-terminal tail was required for S-mediated entry (Fukushi et al., 2005). The tail of S was also inhibitory in mediating entry of retroviruses (Giroglou et al., 2004; Moore et al., 2004). It is likely that the S tail sequence negatively regulates the membrane fusion activity of the S protein, and that in SARS-CoV virions, other

proteins function to activate the S protein membrane fusion activity. Consistent with these earlier reports, we found that the full-length S protein would not pseudotype VSVΔG-EGFP to generate infectious virions, while S protein with its cytoplasmic tail deleted and replaced with an HA tag pseudotyped effectively. Taken together, all evidence indicates that VSVΔG-S is a single-cycle vector.

The strength of the immune response to proteins expressed by replication-competent VSV vectors given i.n. correlates positively with their ability to replicate and spread systemically (Publicover et al.,2005; Simon et al., 2007). Single-cycle vectors, which do not spread systemically (Simon et al., 2007), are relatively poor vectors when given i.n., yet generate strong immune responses when given i.m. (Publicover et al., 2005). In the studies reported here we therefore tested the single-cycle VSVΔG-S vector only by the intramuscular route. We found that one dose of the vector was able to generate high levels of SARS-CoV neutralizing antibody titers of about 1:200. These neutralizing titers were at least ten-fold greater than what was required for complete protection against SARS-CoV replication in mice in our previous study (Kapadia et al., 2005), and two-fold greater than those induced by the replication-competent VSV-S given i.m.. Because antibody responses are sufficient for controlling SARS-CoV infection (Kapadia et al., 2005; Yang et al., 2004), these titers are predictive of protection in the mouse model.

Although the SARS-CoV neutralizing antibody titers obtained from mice immunized i.n. with replication-competent VSV-S were higher (average ~1:600) than the titers obtained from animals immunized i.m., we also know that the replication-competent vectors spread systemically after vaccination by this route (Simon et al., 2007). The virus replicates in the lungs, causes a viremia, and spreads to multiple organs. Such widespread dissemination of the vector could also raise safety concerns.

How can we explain the greater potency of the single-cycle vectors relative to replication-competent vectors given i.m.? First, we have evidence that replication-competent and single-cycle vectors are both effectively single-cycle vectors when given i.m. (Ian Simon, unpublished results). Second, the single-cycle vector may be more effective because of the greater expression of S protein in the absence of the upstream G protein gene. Because of transcriptional attenuation (Iverson and Rose, 1981), the removal of the G gene leads to greater transcription and expression of the SARS-CoV S gene. In order to evaluate this possibility, we quantified the expression of S (treated with PNGase F) by VSV-S and VSVΔG-S relative to N/P expression in the gel shown in Fig. 1D. We found that VSVΔG-S expresses approximately 47% more S protein than VSV-S. Lastly, it is also possible that expression of G protein from the replication-competent vector competes with the S protein for the antibody response.

The results reported here, along with earlier studies showing potent induction of cellular immune responses by single-cycle vectors (Publicover et al., 2005), indicate that these single-cycle vectors are excellent alternatives to live-attenuated VSV vaccine vectors and that they warrant further development.

Material and methods

Plasmids

To construct pVSVΔG-SARS S, the SARS-CoV S gene was amplified from pVSV-SARS S (Kapadia et al., 2005) by PCR using the following primers: 5′-GATCGATCACGCGTAACATGTTTATTTTCTTATTATTTC-3′ and 5′-CGATCCCCCCGGGCTAGCTTATGTGTAATGTAATTTGACACCC-3′. The PCR product was digested with MluI and NheI (sites underlined) and ligated to the purified 12,704 bp fragment resulting from the digestion of pVSVXN2 (Schnell et al., 1996) with the same enzymes.

The plasmid pVSVΔG-EGFP1 expressing EGFP from the first position in the genome was generated by digesting pVSV1XN-EGFP (Ramsburg et al., 2005) with HpaI and XbaI. The ~12-kb vector fragment was purified and ligated to the ~1.4 kb fragment resulting from the digestion of pVSVΔG (Roberts et al., 1999) with the same enzymes. The resulting plasmid was designated pVSVΔG-EGFP1.

pCAAGS-SARS SΔtail-HA was made by PCR amplification of the SARS-CoV S gene with primers that replaced the region encoding the last 28 residues of the carboxy-terminal tail with a sequence encoding the HA epitope tag. The following primers were used: 5′-GATCGATCCTCGAGAACATGTTTATTTTCTTAATTATTTC-3′ and 5′-CGATCCCCCGGGCTAGCTTA**GGCGTAATCTGGGACGTCGTATGGGTA**CTTGAGGCAACTGCAACAACTAGTC-3′. The sequence encoding the HA tag is shown in bold. The resulting PCR product was digested with XhoI and NheI (sites underlined) and ligated into pCAAGS (Niwa et al., 1991) also digested with the same enzymes. Construction of pCAAGS-G was previously described (Okuma et al., 2001).

Recombinant VSV recovery and preparation

Viruses were recovered from plasmids pVSVΔG–SARS S and pVSVΔG–EGFP1 by previously described methods (Schnell et al., 1997). The recovered viral supernatants were then transferred onto BHK-21 cells that had been transfected (described below) with pCAAGS-G (Okuma et al., 2001). The supernatants containing VSVΔG-S and VSVΔG-EGFP1 complemented with G were collected after 36 h. The viruses were titered on BHK-G cells (Schnell et al., 1997) using a standard plaque assay.

To obtain VSVΔG-EGFP1 pseudotyped with the SΔtail-HA protein, we transfected BHK-21 cell with pCAAGS-SARS SΔtail-HA (described below). Transfected cells were infected with recovered VSVΔG-EGFP1 complemented with VSV G. One hour after infection, the input virus was removed and the cells were washed 3 times with phosphate buffered saline (PBS). DMEM containing 5% FBS was added to the cells. The media containing VSVΔG-EGFP1 complemented with SΔtail-HA was collected after 36 h. The virus was titered on Vero E6 cells by assessing the number of cells expressing EGFP.

VSV-SARS S (VSV-S) (Kapadia et al., 2005) and wt VSV (Lawson et al., 1995) recovery were previously described.

Non-complemented VSVΔG-S was obtained by infecting BHK-21 cells with VSVΔG-S at an MOI of 5 for 1 h. The cells were then washed 5 times with PBS to remove any input virus. DMEM with 5% FBS was added to the cells and incubated overnight. The media was collected and subjected to ultracentrifugation for 1 h at 100,000 ×*g* in order to concentrate virus.

Transfections

Nine micrograms of DNA was diluted in 0.6 ml of OptiMEM (Invitrogen, Carlsbad, CA), and 30 μl of Lipofectamine Reagent (Invitrogen, Carlsbad, CA) was also diluted in 0.6 ml of OptiMEM. The DNA and Lipofectamine mixtures were combined and incubated for 30 min at room temperature. BHK-21 cells (2×10^6 cells plated 18 h earlier) were washed with PBS, and 4.8 ml of OptiMEM was added. The DNA/Lipofectamine was added to the cells and incubated at 37 °C for 5 h. Then 6 ml of Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal bovine serum (FBS) was added and left overnight at 37 °C. The next morning the media was replaced with DMEM containing 5% FBS. The transfection was allowed to continue for 48 h after the addition of the DNA/Lipofectamine mixture.

Metabolic labeling

BHK-21 cells were infected with wt VSV, VSV-S or VSVΔG-S at a multiplicity of infection (MOI) of 20. After 5 h the cells were washed twice with methionine-free DMEM and incubated with 100 μCi of [^{35}S]-methionine in 1 ml of methionine-free DMEM for 30 min at 37 °C. The cells were then washed twice with PBS and solubilized with a detergent solution (1% Nonidet P-40, 0.4% deoxycholate, 50 mM

Tris–HCl [pH 8], 62.5 mM EDTA). Lysates were analyzed by SDS-PAGE. The protein samples were treated with Peptide *N*-Glycosidase (PNGase) F (New England Biolabs, Beverly, MA) according to manufacturer's instructions.

Fluorescence microscopy

For indirect immunofluorescence microscopy, BHK-21 cells plated on glass coverslips were infected with either wt VSV or VSVΔG-S. After 6 h the cells were washed twice with PBS and fixed with 3% paraformaldehyde. The cells were then washed twice with PBS containing 10 mM glycine and incubated with serum from a SARS-CoV infected mouse at a dilution of 1:200. The coverslips were washed twice with PBS-glycine and incubated with Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes, Eugene, OR) diluted 1:500. The cells were washed twice with PBS-glycine and mounted on slides. Cells were imaged using a Biorad μ Radiance confocal scanning system on a Nikon Elipse TE300 microscope with a 60× planapochromat objective.

Fluorescence microscopy to visualize EGFP was performed with a Nikon Microphot FX microscope equipped with a 40× planapochromat objective, epifluorescence, and a Spot digital camera.

Vaccination protocol

Ten-week-old BALB/c mice (Charles River Laboratories) were used in this study. Single intranasal inoculations of 5×10^5 plaque forming units (pfu) of wt VSV and VSV-S were administered in a volume of 25 μl to animals lightly anesthetized with 20% Isoflurane (Baxter, Deerfield, IL) diluted in propylene glycol (v/v). Single intramuscular inoculations of 5×10^5 pfu of wt VSV, VSV-S and VSVΔG-S were administered in a volume of 50 μl in the hind leg muscle.

Virus neutralization assays

The VSV neutralization titers are defined as the highest dilution of serum that can completely neutralize infectivity of 100 pfu of VSV on BHK-21 cells. This assay was described previously (Rose et al., 2001).

In order to measure SARS-CoV neutralizing antibodies in serum, VSVΔG-EGFP1/ SΔtail-HA was first incubated with two monoclonal antibodies, I1 and I14 (Lefrancois and Lyles, 1982), at a dilution of 1:1000 per antibody for 1 h at 37 °C to neutralize potential infection due to any residual VSV G that may have been incorporated into the particles pseudotyped with SΔtail-HA protein. Serum samples were serially diluted with DMEM containing 5% FBS. Approximately 75 infectious pseudotyped particles were added to each serum dilution in a final volume of 30 μl. The mixture was incubated for an hour at 37 °C. 25 μl of each dilution was transferred to a monolayer of Vero E6 cells grown in a 96-well plate. After 1 h at 37 °C, 50 μl of DMEM with 5% FBS was added to each well and the cells were incubated for 16 to 20 h at 37 °C. Infection was determined by visualizing EGFP expression using an Olympus CK40 microscope equipped for epifluorescence. Each dilution was measured in duplicate. The titer was determined to be the highest dilution at which both duplicates showed no infection.

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