Recombinant simian varicella viruses expressing respiratory syncytial virus antigens are immunogenic

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Recombinant simian varicella viruses (rSVVs) were engineered to express respiratory syncytial virus (RSV) antigens. The RSV surface glycoprotein G and second matrix protein M2 (22k) genes were cloned into the SVV genome, and recombinant viruses were characterized in vitro and in vivo. rSVVs were also engineered to express the membrane-anchored or secreted forms of the RSV-G protein as well as an RSV G lacking its chemokine mimicry motif (CX3C), which may have different effects on priming the host immune response. The RSV genes were efficiently expressed in rSVV/RSV-infected Vero cells as RSV-G and -M2 transcripts were detected by RT-PCR, and RSV antigens were detected by immunofluorescence and immunoblot assays. The rSVVs replicated efficiently in Vero cell culture. Rhesus macaques immunized with rSVV/RSV-G and rSVV/RSV-M2 vaccines produced antibody responses to SVV and RSV antigens. The results demonstrate that recombinant varicella viruses are suitable vectors for the expression of RSV antigens and may represent a novel vaccine strategy for immunization against both pathogens.

INTRODUCTION

Respiratory syncytial virus (RSV) is a significant pathogen worldwide of young children and the elderly, often causing lower respiratory tract infection and requiring hospitalization. Nearly one-half of all infants are infected with RSV during the first year of life, and nearly 100% are infected by age two, with the incidence of childhood RSV hospitalizations on the rise (Glezen, 2004). RSV is also a major pathogen of the elderly, with mortality in the USA approaching the number that is caused by seasonal influenza (Falsey et al., 2005). The World Health Organization has listed the development of a successful RSV vaccine as a key health priority (Crowe, 1995). Obstacles to RSV vaccination include incomplete immunity even after natural infection, the inefficient immune systems of the very young and elderly target vaccinees, and the legacy of a failed vaccine trial in the 1960s that led to immunopathology and enhanced disease in children (Collins & Murphy, 2002).

The RSV-G (attachment) glycoprotein is a major protective antigen, eliciting strong neutralizing antibody responses and the induction of cytotoxic T-lymphocytes (CTL) (Bukreyev et al., 2006; Collins & Murphy, 2002). RSV G is expressed as secreted and membrane-anchored forms, but the role of each in stimulating protective or detrimental effects is unknown (Johnson & Graham, 1999; Maher et al., 2004). Immunization of animals with recombinant viral vectors expressing RSV G elicits immune protection against RSV infection (Takimoto et al., 2004; Wyatt et al., 1999; Olszewska et al., 2004; Chen et al., 2002). Additionally, the 22 kDa second matrix (M2) protein of RSV elicits strong CD8+ T-cell responses, and when expressed in a recombinant vaccinia virus it induces immunity against RSV in immunized mice (Connors et al., 1992). A successful RSV vaccine will probably include immunogens such as the G and M2 antigens that elicit humoral and CTL immune responses (Van Drunen Littel-Van den Hurk et al., 2007).

Varicella-zoster virus (VZV) is a human alphaherpesvirus that causes chickenpox (varicella), usually during childhood (Cohen et al., 2006). Following resolution of the primary disease, VZV establishes latency in the neural ganglia of the host. Later in life, especially in the elderly, the virus may reactivate to cause shingles (herpes zoster) and postherpetic neuralgia. The live, attenuated VZV Oka vaccine is safe and effective for the prevention of chickenpox in children as young as 12 months of age (Grose, 2005). The VZV vaccine is also effective for the prevention of herpes zoster and postherpetic neuralgia in older individuals (Oxman et al., 2005).

The VZV Oka varicella vaccine is an attractive vector for immunization with foreign antigens for several reasons (Arvin et al., 1999), including (i) the vaccine is sufficiently attenuated to prevent acute varicella disease in most
vaccines, including leukaemic and human immunodeficiency virus (HIV)-infected children (Gershon et al., 1984; Levin et al., 2006), (ii) the 124.8 kbp VZV genome allows the insertion of large coding regions into its DNA, (iii) the attenuated virus is able to replicate at immunization sites, allowing for efficient antigen presentation to the host immune system, and (iv) the virus host range is restricted to humans with no animal reservoir, preventing uncontrollable environmental spread. Antigens from other pathogens, including Epstein–Barr virus (EBV), herpes simplex virus (HSV), hepatitis B virus (HBV) and HIV have been inserted into and expressed by VZV, and these recombinant antigens were immunogenic for both VZV and foreign antigens (Shiraki et al., 1991; Heineman et al., 1995, 2004). Unfortunately, thorough evaluation of recombinant varicella cells is inhibited by the lack of an appropriate animal model.

Simian varicella virus (SVV) is closely related to VZV, and causes varicella and reactivation disease in non-human primates (Gray, 2004). SVV and VZV share a common genomic architecture, antigenic relatedness, and a high degree of homology at the nucleic acid and protein sequence levels (Fletcher & Gray, 1992; Gray et al., 2001). SVV infection of non-human primates serves as a model for the study of VZV pathogenesis and provides a means to test antiviral agents and vaccines that might be applied toward the development of analogous antiviral strategies against VZV.

In this study, recombinant SVVs (rSVVs) expressing RSV-G and -M2 antigens were constructed and analysed. Also constructed were rSVVs expressing secreted or membrane-anchored forms of the RSV-G protein, which may play different roles in priming and influencing the host immune response (Johnson et al., 1998; Johnson & Graham, 1999). An rSVV was also constructed that expressed an RSV-G protein lacking the CX3C fractalkine mimic motif, which may interfere with proper immune cell trafficking to the lungs and/or affect the host immune response phenotype (Harcourt et al., 2006; Tripp et al., 2001). The immunogenicity of the rSVVs expressing the RSV-G and -M2 antigens was evaluated in vivo using a rhesus macaque model. This study shows that recombinant varicella viruses are suitable for the expression of RSV antigens, and may serve as a novel strategy for immunization against both viruses.

**METHODS**

**Cells and viruses.** Vero and CV-1 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5 % newborn bovine serum (NBS), penicillin (5000 U ml$^{-1}$) and streptomycin (5000 U ml$^{-1}$) at 37 °C. Wild-type (wt) SVV and rSVV were propagated on Vero or CV-1 cell monolayers in similar medium with 2 % NBS and antibiotics. RSV strain A2 (ATCC VR-1302) was propagated in Vero cells or HEP-2 cells. Recombinant vaccinia virus expressing RSV G (rRV/RSV-G, a kind gift of Dr Martin J. Cannon, University of Arkansas for Medical Sciences, Little Rock, Arkansas, USA) was propagated on Vero cells. RAW mouse macrophage cells (ATCC TIB 71) were grown in RPMI complete medium supplemented with 10 mM HEPES, 10 mM sodium pyruvate, 1 mM l-glutamine, 10 mM non-essential amino acid solution, 10 % fetal calf serum, 10 000 U penicillin ml$^{-1}$ and 10 000 U streptomycin ml$^{-1}$.

**Construction of rSVV.** Genetic manipulation of the SVV genome was performed by using the SVV cosmid recombination system (Gray & Mahalingam, 2005). For construction of rSVV/RSV-G, the RSV-G coding region was amplified by PCR from the rRV/RSV-G DNA template using primers 5'-ATTGGGATCCCGATAAGTGGTCCGACGAG-3' and 5'-GGGACGCTTCCAGATTATCCTCTCTGG-3', which included BamHI and HindIII restriction sites at the 5' and 3' ends of the gene, followed by cloning into an expression cassette consisting of the human cytomegalovirus (HCMV) immediate-early promoter followed by an SV40 polyadenylation (pA) signal sequence. A KpnI fragment, containing the HCMV-RSV-G-pA, was cloned into a unique KpnI restriction site (nt 20422) within the SVV glycoprotein C gene of SVV cosmid A. The recombinant cosmid DNA was packaged into phage heads (MaxPlax; Epicentre), transduced into *Escherichia coli* strain Epi305 and clones were selected on Luria–Bertani agar plates containing ampicillin and/or chloramphenicol. Recombinant cosmid DNA was harvested using a commercial midiprep kit (Qiagen). Co-transfection of Vero cell monolayers with cosmid A-HCMV-RSV-G DNA and cosmids B, C and D using Superfect reagent (Qiagen) yielded infectious rSVV by days 10–14 post-transfection.

A similar approach was employed to construct rSVV expressing other forms of the RSV-G antigen, rSVV/RSV-G$m_\text{s}$, which expresses only the secreted form of the RSV-G antigen, was generated using primers 5'-CACAGGATCCATCTGGAATG-3' and 5'-GCCAAAGTCTCCTGATATTGTCATTGTTG-3', to amplify the G coding region beginning near the downstream AUG start codon at nt 142 in the RSV-G gene, and to engineer BamHI and HindIII sites onto the ends of the gene. rSVV/RSV-G$m_\text{a}$, expressing only the membrane-anchored form of RSV G, was constructed by site-directed mutagenesis using primers 5'-CATACTATGATTCTGGGATGAGA-3' and 5'-CAATTATAAAGGATGATTGATTCC-3', which engineer BamHI and HindIII sites onto the ends of the gene. Co-transfection of the Vero cells with cosmid A-HCMV-RSV-G DNA and cosmids B, C and D using Superfect reagent (Qiagen) yielded infectious rSVV by days 10–14 post-transfection.

rSVV/RSV-G$m_\text{s}$, expressing the M2-1 RSV protein, was constructed by using the pCMV-Tag2 vector (Stratagene). The HCMV-flag-pA expression cassette was amplified and cloned into the pScript vector. A NotI restriction endonuclease cleavage site was inserted in the multiple cloning site (MCS) of this vector was removed via insertion of a double-stranded adaptor made with the oligonucleotides 5'-AAATAGGGGCGATCATTG-3' and 5'-AAATTGAGGGCGCTCATTAC-3', which engineer BamHI and HindIII sites onto the ends of the gene. rSVV/RSV-G$m_\text{s}$, expressing the M2-1 RSV protein, was constructed by using the pCMV-Tag2 vector (Stratagene). The HCMV-flag-pA expression cassette was amplified and cloned into the pScript vector. A NotI restriction endonuclease cleavage site was inserted in the multiple cloning site (MCS) of this vector was removed via insertion of a double-stranded adaptor made with the oligonucleotides 5'-AAATAGGGGCGATCATTG-3' and 5'-AAATTGAGGGCGCTCATTAC-3', which engineer BamHI and HindIII sites onto the ends of the gene. rSVV/RSV-G$m_\text{s}$, expressing the M2-1 RSV protein, was constructed by using the pCMV-Tag2 vector (Stratagene). The HCMV-flag-pA expression cassette was amplified and cloned into the pScript vector. A NotI restriction endonuclease cleavage site was inserted in the multiple cloning site (MCS) of this vector was removed via insertion of a double-stranded adaptor made with the oligonucleotides 5'-AAATAGGGGCGATCATTG-3' and 5'-AAATTGAGGGCGCTCATTAC-3', which engineer BamHI and HindIII sites onto the ends of the gene. rSVV/RSV-G$m_\text{s}$, expressing the M2-1 RSV protein, was constructed by using the pCMV-Tag2 vector (Stratagene). The HCMV-flag-pA expression cassette was amplified and cloned into the pScript vector. A NotI restriction endonuclease cleavage site was inserted in the multiple cloning site (MCS) of this vector was removed via insertion of a double-stranded adaptor made with the oligonucleotides 5'-AAATAGGGGCGATCATTG-3' and 5'-AAATTGAGGGCGCTCATTAC-3', which engineer BamHI and HindIII sites onto the ends of the gene.
PCR and DNA sequence analysis were used to confirm the presence of the RSV genes within the rSVV DNA isolated from infected cells. PCR conditions included an initial denaturation step of 94 °C for 2 min, followed by 30 cycles of 94 °C for 1 min, 55 °C for 3 min, 72 °C for 2 min and a final extension step of 72 °C for 7 min. PCR products were cloned into the pGEM-T Easy vector and sequenced using primers to the SP6 and T7 promoters.

Analysis of transcription by RT-PCR. Total cellular RNA was isolated from infected cells by using RNA-Bee (Tel-Test), and DNase treated (Ambion DNA-Free; Ambion). Transcription of RSV genes was confirmed by RT-PCR (Access-Quick RT-PCR System; Promega) using RSV-G primers 5'-GTGTGGATGTGGTGGCTCTC-3' and 5'-CATCATATTGATATGCTCCG-3' and RSV-M2 primers 5'-CTTGGGATCTATCTAAGG-3' and 5'-GTATGGAAGCTTTACTACAAGG-3'. RT-PCR conditions were 45 °C for 45 min for reverse transcription, an initial denaturation step of 94 °C for 2 min, followed by 36 cycles of 94 °C for 30 s, 60 °C for 30 s, 68 °C for 3 min and a final extension step of 68 °C for 5 min.

Virus growth assays. Cell-free SVV preparations were generated as follows: SVV-infected Vero cell monolayers were scraped from three 175 cm² flasks into 30 ml medium. Infected cells were pelleted and then resuspended in 10 ml DMEM supplemented with 10% FCS and 10% DMSO. Suspended cells were sonicated on ice for one 15 s pulse and then one 30 s pulse. Samples were centrifuged at 1800 g for 15 min at 4 °C and the supernatant was frozen at −70 °C. Virus titre was determined by plaque assay on CV-1 cells. For growth assays, CV-1 duplicate cell monolayers (25 cm²) were infected with 800 p.f.u. of each virus per flask, and virus titres were calculated by plaque assay on CV-1 cells at 0, 8, 24, 48 and 72 h post-infection (p.i.). Viral plaque diameters were measured under light microscopy at 72 h p.i. At least 20 plaques for each virus were measured and statistical significance was determined by Student's t-test.

Detection of viral antigens in rSVV/RSV-infected cells. Immunoblot and immunofluorescence analyses were used to confirm expression of the RSV-G and -M2 antigens in rSVV-infected Vero cells. For immunoblot analysis, protein lysates of SVV-infected Vero cells in solubilization buffer (25 mM Tris/HCl, 250 mM NaCl, 5 mM EDTA, 0.5% deoxycholate, 1.0% Triton X-100) including protease inhibitors were fractioned by SDS-PAGE on 10% gels and then transferred to PVDF membranes. RSV antigens were detected by chemiluminescence using primary antibodies [goat polyclonal anti-RSV (AB1128; Chemicon) or anti-FLAG monoclonal antibody (mAb; Stratagen)], secondary horseradish peroxidase (HRP)-conjugated antibodies (rabbit anti-goat IgG or goat anti-mouse IgG) and chemiluminescence substrate (Pierce). Membranes were applied to Kodak BioMax XAR film (Fisher) and developed to visualize protein bands.

For immunofluorescence, rSVV-infected Vero cells were seeded onto coverslips and fixed with methanol or paraformaldehyde. In some cases infected cells were permeabilized with 0.2% Triton X-100 in PBS for 10 min or treated with Brefeldin A (10 μg ml⁻¹) for 12–16 h prior to cell fixation. RSV-G and -M2 antigens were detected using primary antibodies [goat polyclonal anti-RSV, mAb to RSV G (mAb 8593; Chemicon) or anti-FLAG mAb] and secondary fluorescein-isothiocyanate (FITC)-conjugated antibodies (rabbit anti-goat IgG or goat anti-mouse IgG). Immunofluorescence was visualized using an Olympus BX40 microscope equipped with a digital camera.

Chemotaxis assay. An adapted chemotaxis assay was used to determine recruitment of RAW mouse macrophages (Gevrey et al., 2005). Culture media from RSV-, rSVV/RSV-G-, rSVV/RSV-Gp-, wt SVV- or mock-infected CV-1 cells were concentrated using Amicon Ultra-15 filters (Millipore) by centrifugation at 3200 g for 15 min at 4 °C, excluding low molecular mass (<50 kDa) proteins. Concentrated medium (50 μl) and 750 μl DMEM were added to each well of a 24-well culture dish. Cell culture inserts (Falcon) with 8 μm filters were placed in the wells and 5 × 10⁵ serum-starved RAW cells in 200 μl DMEM were added. After incubation at 37 °C for 4 h in a CO₂ incubator, inserts were removed and cells in the lower chamber were centrifuged and resuspended in 100 μl DMEM. Cells in samples were counted using a haemocytometer and the total number of cells recruited to the lower well was calculated.

Animal immunizations with rSVV. Studies involving animals were conducted at the Tulane National Primate Research Center in compliance with the Public Health Service policy on humane care and use of laboratory animals. Rhesus macaque monkeys (Macaca mulatta) were confirmed to be seronegative to SVV by serum neutralization assay prior to experimental infection. A group of five animals, designated DB31, DN76, EK04, EP23 and FH95, was intratracheally and subcutaneously infected on day 0 with 8 × 10⁵ p.f.u. of each rSVV/RSV-G and rSVV/RSV-M2 in infected Vero cells. A second group of five animals, designated DE36, DH08, DR47, FD34 and FH96, was infected in a similar manner with rSVV vaccines expressing the simian immunodeficiency virus (SIV) env and gag antigens and served as a negative-control group. Booster immunizations with the same virus titres were administered on days 35 and 70 p.i. Clinical and virological parameters of SVV infection were evaluated as described previously (Gray et al., 1998; Gray, 2003). Viraemia was monitored by harvesting peripheral blood mononuclear cells using Ficoll-Hypaque gradients and determining SVV titres by co-culture on Vero cell monolayers. Skin rash was scored on an established scale of 0 to 4 + , with no rash = 0 and a severe rash = 4 + . Hepatitis was assessed by aspartate transaminase assay of animal blood specimens.

Neutralizing antibody titres to RSV and SVV were determined by serum neutralization of cell-free virus as the serum dilution that reduced RSV or SVV viral plaque counts by 50% on CV-1 cell monolayers (Ou et al., 2007). Enzyme-linked immunosorbent (ELISA) assay was also used to quantify antibody responses to SVV and RSV antigens. Total protein lysates from SVV- or RSV-infected Vero cells were used to coat 96-well-polystyrene plates and dilutions of animal sera were applied. After 3 h incubation, plates were washed and secondary anti-mouse IgG alkaline-phosphatase-conjugated antibody (Sigma) was added and allowed to adsorb for 1 h. Substrate (SigmaFast pNPP tablets; Sigma) was added for 30 min and plates were read at 405 nm in a microplate ELISA reader.

RESULTS

Generation of rSVV/RSV-G and rSVV/RSV-M2

A cosmid recombinant system was used to construct rSVV expressing the RSV-G and -M2 genes (Gray & Mahalingam, 2005). The coding regions for RSV G or RSV M2 were PCR amplified and cloned into expression cassettes under the control of the HCMV immediate-early promoter. A FLAG tag sequence was added to the 5’ end of the RSV-M2 gene to aid in protein detection. The HCMV- RSV-G-pA cassette was ligated into the unique KpnI restriction site in SVV ORF 14 (glycoprotein C) within cosmids A, while the HCMV-RSV-M2-pA cassette was inserted into an engineered Ascl site in the intergenic region between ORFs 12 and 13 within cosmid A.

The presence of the RSV-G gene within the rSVV genome was confirmed by PCR detection within total cell DNA.
isolated from rSVV/RSV-G-infected Vero cells (Fig. 1a). RSV-G transcripts were detected within rSVV-infected cells as confirmed by RT-PCR (Fig. 1b). RSV-G antigen expression within rSVV-infected Vero cells, but not wt SVV-infected cells, was confirmed by immunofluorescence using a polyclonal antibody specific for RSV antigens (Fig. 1c) or when using an RSV-G-specific mAb (data not shown). RSV-G antigen expression was confirmed by immunoblot analysis using RSV-specific polyclonal antibody. An ~90 kDa band corresponding to the full-length RSV-G protein was detected in Vero cells infected with rSVV/RSV-G (Fig. 1d, lanes 2, 3), a recombinant vaccinia virus expressing RSV G (lane 4) and wt RSV (lane 5), but not in wt SVV-infected cells (lane 1).

Similarly, the presence of the RSV-M2 gene was detected within the rSVV/RSV-M2 genome by PCR (Fig. 2a) and M2 transcripts were detected in infected Vero cells by RT-PCR (Fig. 2b). Expression of the 22 kDa M2 antigen in rSVV/RSV-M2-infected Vero cells, but not wt SVV-infected cells, was confirmed by immunofluorescence and immunoblot analysis using anti-FLAG antibody (Fig. 2c, d). The RSV-G and -M2 genes were stably expressed over multiple passages of the rSVVs in cell culture.

**rSVV in vitro replication**

The *in vitro* growth properties of rSVV/RSV-G and rSVV/RSV-M2 were analysed to determine the effect of insertion of the RSV genes into the SVV genome. CV-1 cells were infected with 800 p.f.u. cell-free rSVV/RSV-G, rSVV/RSV-M2 or wt SVV and viral titres were determined at various times p.i. rSVV expressing RSV G replicated as efficiently as wt SVV in CV-1 cell culture (Fig. 3a). rSVV/RSV-M2 replicated *in vitro*, but at 72 h p.i. rSVV/RSV-M2 had a significant, almost 10-fold, reduction in viral titre (4.7 ± 0.4 × 10⁴ p.f.u.) compared with wt SVV.
immunoblot analysis employing polyclonal RSV antiserum was used. An ~90 kDa protein, corresponding to full-length RSV G, was detected in protein lysate derived from rSVV/RSV-G\textsubscript{M2}-infected Vero cells (Fig. 4a, lane 2). In contrast, a smaller ~80 kDa protein, corresponding to the predicted size of the truncated secreted form of RSV G, was detected in lysate derived from rSVV/RSV-G\textsubscript{G\textsubscript{G}}-infected Vero cells (Fig. 4a, lane 1). Previous studies have shown similar molecular mass differences of membrane-anchored versus secreted RSV G forms (Arnold et al., 2004).

Immunofluorescence analysis was used to confirm the immunoblot data and to localize the RSV G forms in rSVV/RSV-G\textsubscript{G\textsubscript{G}}- and rSVV/RSV-G\textsubscript{M2}-infected Vero cells. Infected cells were fixed with paraformaldehyde to maintain cell architecture. In some cases cells were

Analysis of rSVV expressing secreted and membrane-anchored forms of the RSV-G antigen

The roles of the membrane-anchored and secreted forms of the RSV G in stimulating the immune responses to RSV are unclear. As an initial step toward understanding their potential use in RSV vaccines, rSVV expressing only the RSV-G secreted form (rSVV/RSV-G\textsubscript{s}) or only the RSV-G membrane-anchored form (rSVV/RSV-G\textsubscript{M2}) were constructed using the SVV cosmid genetic system. rSVV/RSV-G\textsubscript{s} was engineered to truncate the 5' end of the RSV-G coding region so that the AUG codon at nt 142 is the first initiation codon. Conversely, rSVV/RSV-G\textsubscript{M2} was engineered via site-directed mutagenesis of the downstream nt 142 AUG codon to GGG, coding for a glycine residue rather than an initiator methionine. RSV-G gene expression was initially confirmed by RT-PCR detection of the RSV-G transcripts in rSVV/RSV-G\textsubscript{s} and rSVV/RSV-G\textsubscript{M2}-infected Vero cells (data not shown).

To verify that the rSVVs expressed either the full-length membrane or the truncated secreted form of RSV G, immunoblot analysis employing polyclonal RSV antiserum was used. An ~90 kDa protein, corresponding to full-length RSV G, was detected in protein lysate derived from rSVV/RSV-G\textsubscript{M2}-infected Vero cells (Fig. 4a, lane 2). In contrast, a smaller ~80 kDa protein, corresponding to the predicted size of the truncated secreted form of RSV G, was detected in lysate derived from rSVV/RSV-G\textsubscript{G\textsubscript{G}}-infected Vero cells (Fig. 4a, lane 1). Previous studies have shown similar molecular mass differences of membrane-anchored versus secreted RSV G forms (Arnold et al., 2004).

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permeabilized with Triton X-100 to facilitate antibody penetration for the detection of intracellular antigen. RSV G was detected with primary anti-RSV antibody followed by a FITC-labelled secondary antibody.

RSV G was detected in non-permeabilized RSV- (Fig. 4b, panel i) and rSVV/RSV-G-/G-infected Vero cells (panel iii), confirming that the wt RSV G as well as the membrane-anchored form of RSV G are present in the cell membrane. The secreted form of RSV G was not readily detected in non-permeabilized rSVV/RSV-Gα-infected cells (panel iv), but was detected in permeabilized cells that were treated with the secretory pathway inhibitor Brefeldin A (panel v), indicating that this form of RSV G was secreted and not retained in the cell membrane. Analysis of negative-control wt SVV-infected cells confirmed that these cells do not express antigens cross-reactive to RSV G (panel ii).

rSVV/RSV-Gp expression of RSV G lacking the fractalkine CX3C motif

The RSV-G protein contains a CX3C (CWAIC, aa 183–187) chemokine-like motif, which functionally mimics fractalkine, a chemokine involved in immune cell recruitment (Tripp et al., 2001), and in maintenance and amplification of Th1 immune responses (Fratricelli et al., 2001). As a first step to investigate the role of this CX3C motif, site-directed mutagenesis was used to engineer a recombinant virus (rSVV/RSV-Gp) that expresses RSV G with a C187R mutation. DNA sequence analysis confirmed the RSV-G mutation within the rSVV/RSV-Gp genome. Expression of the mutated RSV-G gene in rSVV/RSV-Gp-infected Vero cells was confirmed by the detection of RSV-G transcripts employing RT-PCR (data not shown) and RSV-G antigen by immunofluorescence (Fig. 4b, panel vi). A chemotaxis assay was used to determine the functional effects of this mutation. Concentrated proteins derived from culture media of wt RSV or rSVV/RSV-Gp-infected Vero cells effectively recruited RAW mouse macrophages, cells known to be chemotactic toward both fractalkine and the G protein of RSV (Fig. 5) (Gevrey et al., 2005). However, culture medium derived from rSVV/RSV-Gp-infected Vero cells did not exhibit this activity, and cell recruitment was reduced to the same level exhibited by wt SVV. This result indicates that the CX3C→CX3R mutation in RSV G expressed by rSVV/RSV-Gp abrogates its chemotactic ability to mimic fractalkine.

Immunogenicity of rSVV/RSV vaccines

The ability of rSVV to induce immune responses to RSV and SVV antigens was determined in immunized rhesus macaque monkeys. The animals were infected three times over a 70 day period with both rSVV/RSV-G and rSVV/RSV-M2 by intratracheal and subcutaneous inoculation. Although intratracheal inoculation is not practical for vaccination, this approach was used to deliver a precise viral dose to ensure viral infection for the induction of immune responses. The animals did not exhibit the typical signs of acute simian varicella such as skin rash and hepatitis (data not shown). Analysis by serum neutralization assay revealed that four of five rSVV/RSV-immunized animals generated neutralizing antibodies to RSV (Table 1). None of the four control monkeys immunized with rSVV vaccines expressing SIV env and gag antigens, which were tested, generated neutralizing antibodies to RSV. ELISA analysis confirmed that rSVV/RSV-immunized animals generated antibodies to the RSV antigens, while the rSVV/SIV-immunized animals did not (Fig. 6a). All rSVV/RSV-immunized animals developed antibodies to SVV antigens as confirmed by serum neutralization assay and ELISA (Table 1 and Fig. 6b). These results demonstrate that the recombinant varicella vaccines induce antibody responses to the SVV and RSV antigens.

DISCUSSION

This study demonstrates that RSV genes can be incorporated into and expressed from varicella viruses and that the recombinant viruses replicate in cell culture and are immunogenic, inducing humoral immune responses to the SVV and RSV antigens in immunized animals. A recombinant varicella-RSV vaccine could be beneficial for the populations that are most susceptible to VZV and RSV diseases. Vaccination with a recombinant varicella vaccine expressing RSV antigens may be useful for the elderly, who are at risk for both herpes zoster and severe RSV respiratory disease. Among the elderly, RSV is responsible for 18% of doctor’s office visits for respiratory illness and over 10% of hospitalizations for pneumonia, with 78% of RSV respiratory or circulatory associated deaths occurring in persons aged 65 years or older.
Recombinant SVV-RSV vaccines

Table 1. Serum neutralization titres of rSVV-immunized animals

<table>
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<tr>
<th>Animal</th>
<th>Vaccines</th>
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<th>Day 53</th>
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<td>1:10</td>
<td>1:40   (1:80)</td>
<td>1:20 (1:40)</td>
</tr>
<tr>
<td>6</td>
<td>rSVV-SIV</td>
<td>&lt;1:10</td>
<td>&lt;1:10</td>
<td>&lt;1:10</td>
<td>ND</td>
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</tr>
<tr>
<td>7</td>
<td>rSVV-SIV</td>
<td>&lt;1:10</td>
<td>ND</td>
<td>&lt;1:10</td>
<td>ND</td>
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</tr>
<tr>
<td>8</td>
<td>rSVV-SIV</td>
<td>&lt;1:10</td>
<td>ND</td>
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<td>ND</td>
<td>&lt;1:10</td>
</tr>
<tr>
<td>9</td>
<td>rSVV-SIV</td>
<td>&lt;1:10</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>&lt;1:10</td>
</tr>
</tbody>
</table>

*Days after the third immunization.
†Dilution of sera that reduced the number of RSV plaques by 50%.
‡Dilution of sera that reduced the number of wt SVV plaques by 50% (in parentheses).
§Control animals immunized with rSVV expressing SIV env and gag (Ou et al., 2007).
ND, Not determined.

(Thompson et al., 2003; Zambon et al., 2001; Falsey et al., 2005). A recombinant VZV-RSV vaccine might also be effective against childhood chickenpox and RSV infection, although infants younger than 1 year are most vulnerable to severe RSV respiratory disease, while the varicella vaccine is currently only approved for children at least 12 months of age. However, 20–38% of childhood RSV related deaths occur after 1 year (Fleming et al., 2005; Shay et al., 2001). In addition, 71% of children with RSV that caused acute otitis media, the most common paediatric disease, are older than 1 year (Patel et al., 2007). Therefore, immunization of children 1 year and older with an effective recombinant varicella-RSV vaccine could reduce RSV morbidity and mortality and also lower the overall viral burden in the population, thus reducing the frequency of RSV exposure to susceptible infants.

The varicella vaccine, currently administered as one or two subcutaneous inoculations, as well as natural VZV infection, induces a Th1-biased immune response (Sasaki et al., 2003; Kamiyama et al., 2000; Phumiamorn et al., 2003; Yu et al., 2005). Intranasal VZV Oka immunization, which is also feasible, would also probably induce a mucosal Th1-mediated immune response (Bogger-Goren et al., 1982; Terada et al., 2002). The immunopathology and vaccine enhanced disease associated with a failed clinical trial of a formalin inactivated RSV vaccine during the 1960s as well as other RSV vaccines has been related to a detrimental Th2 immune response (Openshaw & Tregoning, 2005). Therefore, a recombinant VZV vaccine expressing RSV antigens may prime the immune response toward a protective Th1-mediated immune response and avoid the immunopathology associated with harmful Th2 responses.

The rSVV/RSV-G and rSVV/RSV-M2 vaccines, administered together, were immunogenic, eliciting humoral immune responses, including neutralizing antibodies to SVV and RSV antigens in immunized rhesus monkeys. While antibody titres were not robust, previous studies with recombinant Sendai virus expressing the RSV-G protein elicited similar antibody titres that offered protection against RSV challenge in a cotton-rat model (Takimoto et al., 2004). It is likely that the rSVV/RSV vaccines would induce stronger immune responses in

Fig. 6. Antibody responses to RSV and SVV antigens in immunized animals. ELISA was used to determine antibody titres to RSV (a) and SVV (b) antigens at various days following the third immunization with rSVV/RSV. Animal FH96 (C) in (a) was immunized with rSVV-SIV. Monkey serum was diluted 1:40 for RSV antibody detection and 1:2000 for SVV antibody detection.

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African green or vervet ( Chlorocebus aethiops ) monkeys, based on a previous study in which vervet monkeys were immunized with rSVV expressing SIV env and gag antigens ( Ou et al. , 2007). Vervet monkeys are most commonly used for simian varicella studies and SVV replicates more efficiently in vervet cells than in rhesus monkey cells ( Gray et al. , 1998 ).

The construction of rSVV expressing various forms of the RSV G provides an opportunity to dissect the relative importance of these RSV G forms in pathogenesis and for inclusion in RSV vaccines. rSVVs were engineered to express either the membrane-anchored (rSVV/RSV-Gm) or secreted (rSVV/RSV-Gs) forms of the RSV-G protein, which may have different effects on priming of the host immune system. It is postulated that the secreted form of the RSV-G protein may be taken up by antigen-presenting cells and presented to CD4 + T-cells, allowing it to prime Th2 subsets of responder cells and promoting a Th2-biased immune response ( Johnson et al. , 1998; Johnson & Graham, 1999 ). Conversely, the membrane-anchored G antigen may be processed as an intracellular antigen and presented to CD8 + T-cells, the induction of which has been shown to prevent or dampen Th2-biased responses via IFN-γ production ( Srikiatkhachorn & Braciale, 1997 ). Indeed, an engineered RSV lacking the secreted form of the G glycoprotein was attenuated in mice, but induced protection against RSV challenge ( Maher et al. , 2004 ), suggesting that the secreted RSV-G protein plays a role in viral pathogenicity and is not essential for efficient induction of protective immune responses. The rSVV generated in this study will allow analysis of the advantages of membrane-anchored opposed to secreted G proteins for immunization in a varicella vector. Also constructed was rSVV/RSV-Gp that expresses an RSV-G protein lacking the CX3C fractalkine mimic motif, which may interfere with immune cell recruitment to the lungs ( Harcourt et al. , 2006; Tripp et al. , 2001 ). Since fractalkine serves as a maintenance and amplification circuit for Th1 responses, RSV may use this motif in the G glycoprotein to co-opt or interrupt this response, perhaps aiding in the Th2 response favoured by the virus. In this study, a chemotaxis assay confirmed the ability of RSV G, expressed in a SVV vector, to serve as a chemo-attractant for mouse macrophages and demonstrated that change of a single amino acid within the fractalkine mimic motif abrogates this effect. In a future study, the immune responses of animals immunized with SVV/RSV-G or SVV/RSV-Gp can be compared to assess whether deletion of the fractalkine mimic motif promotes a Th1-biased immune response against RSV.

This study provides proof-of-principle that recombinant varicella virus is a suitable vector for the expression of RSV antigens and may be useful as a combination vaccine to protect against both pathogens. Future studies will evaluate the ability of rSVV/RSV vaccines to protect immunized vervet monkeys against RSV respiratory disease and vaccine-enhanced disease following RSV challenge using an established simian RSV infection model, which simulates human RSV respiratory infection and has been used to evaluate candidate RSV vaccines ( Byrd & Prince, 1997; Jin et al. , 2003 ).

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Recombinant SVV-RSV vaccines

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