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Respiratory syncytial virus (RSV) fusion protein expressed by recombinant Sendai virus elicits B-cell and T-cell responses in cotton rats and confers protection against RSV subtypes A and B

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Abstract

The respiratory syncytial virus (RSV) is a serious pediatric pathogen for which there is currently no clinically-approved vaccine. This report describes the design and testing of a new RSV vaccine construct (rSV-RSV-F), created by the recombination of an RSV F sequence with the murine parainfluenza virus type 1 (Sendai virus, SV) genome. SV was selected as the vaccine backbone for this study, because it has previously been shown to elicit high-magnitude, durable immune activities in animal studies and has advanced to human safety trials as a xenogenic vaccine for human parainfluenza virus-type 1 (hPIV-1). Cells infected with the recombinant SV expressed RSV F protein, but F was not incorporated into progeny SV virions. When cotton rats were inoculated with the vaccine, high-titer RSV-binding and neutralizing antibodies were induced as well as interferon- γ -producing T-cells. Most striking was the protection against intra-nasal RSV challenge conferred by the vaccine. The rSV-RSV-F construct was also tested as a mixture with a second SV construct expressing the RSV G protein, but no clear advantage was demonstrated by combining the two vaccines. As a final analysis, the efficacy of the rSV-RSV-F vaccine was tested against an array of RSV isolates. Results showed that neutralizing and protective responses were effective against RSV isolates of both A and B subtypes. Together, experimental results encourage promotion of this recombinant SV construct as a vaccine candidate for the prevention of RSV in humans.

Keywords

Respiratory Syncytial Virus Vaccine; neutralizing antibody; Sendai virus reverse genetics

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INTRODUCTION

Respiratory syncytial virus (RSV) is the leading cause of hospitalizations for viral respiratory infections in infants and young children. In the United States, an estimated 70,000 to 125,000 infants are hospitalized annually with RSV pneumonia or bronchiolitis. Worldwide, RSV is estimated to cause more than 900,000 deaths per year. Clinical observations indicate that the first infection is generally the most severe, whereas subsequent infections tend to be milder. Such observations underscore the need for the design and development of an effective RSV vaccine [1–3].

RSV vaccine studies began more than four decades ago. The most notable program culminated in a 1960s pediatric clinical trial in which RSV cultures were inactivated with formalin and administered to children. Unfortunately, this vaccine exacerbated disease when children were subsequently infected by RSV upon natural exposure [4]. It is now proposed that the formalin inactivated RSV vaccine elicited little neutralizing antibody, explaining the lack of protection. Moreover, in subsequent research studies, animals inoculated with the formalin-inactivated vaccine and challenged with RSV experienced severe lung inflammatory responses characterized by a skewed CD4⁺ T-cell response (in the absence of neutralizing antibodies) and the influx of eosinophils in the lung [5–11]. The importance of B-cell responses to protection has been demonstrated by a number of passive protection studies using RSV-neutralizing immune globulin and humanized monoclonal antibodies [1;12–14].

Interest in eliciting both humoral and cellular immune responses has spurred examination of live attenuated vaccine vectors. RSV and PIV vaccine candidates include cold-adapted or host-range-restricted viruses in unmodified or recombinant form [15–18]. Various subunit, fusion protein or peptide vaccines have also been tested in the RSV field. Thus far, there has been no indication of a clear clinical vaccine success, either because (i) safety problems have surfaced, (ii) the immunogenicity of vaccines has been inadequate, or (iii) the studies have not reached completion. The challenge that remains is to strike an effective balance between the safety and immunogenicity of current RSV candidate vaccines [19–24].

In our laboratory, studies with SV have highlighted the strengths of this vaccine vector for clinical applications. Initially, we demonstrated that African green monkeys were consistently protected against challenge with the human cognate virus hPIV-1 following vaccination with SV [25]. We next advanced SV to clinical phase I trials and showed that intranasal application of the vaccine was well tolerated in a cohort of healthy adults, all of whom were sero-positive [26]. The utility of unmodified SV as a naturally attenuated vaccine candidate for human PIV-1 prompted us to investigate SV as a platform for other immunogens.

Here we describe the preparation and evaluation of a novel recombinant SV vaccine expressing the RSV F protein. We show that the vaccine elicits RSV-specific neutralizing antibody and T-cell responses in cotton rats. Importantly, the vaccine also confers protection against RSV infections, even when the challenge virus is mismatched with vaccine by origin, sequence and subtype. Results strongly encourage advancement of the SV-based respiratory virus vaccine approach to clinical trial.

MATERIALS AND METHODS

Construct design

Replication-competent recombinant SV was rescued from the full genome SV cDNA containing an RSV F gene (for expression of a membrane-anchored form of the F protein) by a reverse genetics system using a modification of previously described methods [27]. To create a recombinant SV that produced the membrane-anchored form of RSV F protein, the full-length

cDNA of SV (Z strain) [28] was modified to create a unique *NotI* site in the non-coding region between the F and HN genes (pSV(+)*N* [29], Figure 1A). Viral RNA was extracted from RSV strain A-2 (American Type Culture Collection, Rockville, MD)-infected HEp-2 cells and the F gene was amplified by reverse-transcription (RT)-PCR (Titan One Tube System; Roche). The PCR forward primer included a *NotI* site and the reverse primer included an SV transcription termination signal, an intergenic (IG) sequence CTT, a transcription initiation signal and a second *NotI* site (see Figure 1A). Both RSV F cDNA and pSV(+)*N* were cleaved with *NotI* for recombination.

To rescue the recombinant virus, we infected 293T cells with a UV-inactivated, T7 RNA polymerase-expressing recombinant vaccinia virus (vTF7.3) for 1 hr at 37 °C at a multiplicity of infection (MOI, defined prior to UV treatment) of 3. Cells were co-transfected with the F-containing SV cDNA plasmid (1 µg) with three supporting T7-driven plasmids expressing the NP, P, or L genes of SV (1 µg pTF1SVNP, 1 µg pTF1SVP, and 0.1 µg pTF1SVL) in the presence of Lipofectamine (8 µl; Life Technologies, Grand Island, NY). Cells were then incubated for 40 hr. Cell lysates were subsequently prepared and inoculated into 10-day-old embryonated hens eggs. Allantoic fluids were harvested after 72 hr and virus was cloned by plaque purification on LLC-MK2 cells. Cloned recombinant SV was amplified once more in embryonated eggs to prepare vaccine stocks. Recovered virus was designated rSV-RSV-F.

Immunoprecipitation of F protein from infected HEp-2 cells

To check for RSV F protein expression by rSV-RSV-F infected cells, HEp-2 cells in 6 well plates were infected with the recombinant virus or with RSV-A2 for 1 hr at room temperature. Infected cells were then cultured for 2 days at 34°C and labeled with ³⁵S-Trans (100 µCi/ml) overnight at 34°C. Cells were lysed with 1 ml of TNE buffer (10 mM Tris, pH 7.4; 150 mM NaCl; 0.5% NP-40). Supernatants were clarified by a high-speed spin (15,000 × g, 15 min). Labeled RSV F proteins in the clarified supernatants were captured by mixing with a murine RSV-F-specific monoclonal antibody (Clone 631, Cat#C65063, Biodesign Intl. Saco, ME) bound by sheep anti-mouse IgG Dynal Beads (M-280, cat#112.01, Dynal Biotech, Lake Success, NY). The immunocomplexes were resolved by SDS-PAGE (10% gels) and analyzed with Kodak BioMax MR film.

Purification and characterization of recombinant SV particles

HEp-2 cells were infected with recombinant SV at an MOI of 5 and harvested after incubation for 3 days at 34°C. Recombinant SV particles were purified from the cell supernatants by centrifugation on a sucrose gradient. Purified virus (quantified for protein content using a Micro BCA™ Protein Assay Kit, Pierce, Rockford, IL) was processed on an SDS gel and stained with GelCode Blue Stain Reagent. Additional gels were processed for Western blotting as described below. RSV-infected cell lysates were used as positive controls.

To obtain the control, wild-type RSV-infected cell lysate, HEp-2 cells were infected with RSV-A2 in PBS at room temperature for 1 hour. The inoculate was removed and cells were cultured in Dulbecco's modified eagle's medium (Cambrex Bio Science Walkersville, Inc, Walkersville, MD) supplemented with glutamine, gentamicin and 10% fetal calf serum. Cells were harvested on day 3 and lysed with 0.2 ml of TNE buffer (10 mM Tris [pH 7.4], 150 mM NaCl, 0.5% NP-40, and 1 mM EDTA). Supernatants were then clarified by centrifugation (15,000 × g, 10 min).

Western blot analyses

Proteins were separated by SDS-polyacrylamide gel electrophoresis under non-reducing conditions (to preserve antigenic determinants) and transferred to an Immobilon membrane (Millipore, Danvers, Mass.). Membranes were treated with 5% milk/TBST (Tris buffered

saline plus 0.5% Tween), incubated with an RSV F-specific monoclonal antibody (antibody 1269, kindly provided by Dr. Coelingh, NIAID [30]), washed again, and incubated with a goat anti-mouse IgG (H+L) horse radish peroxidase (HRP) conjugate (BioRad, Hercules, CA). A final reaction was with a SuperSignal West Pico Chemiluminescent (HRP) Substrate (Pierce, Rockford, IL) used as recommended by the manufacturer.

Animals and Immunizations

Groups of 5 adult female cotton rats (*Sigmodon hispidus*; Harlan Sprague Dawley, Indianapolis, IN) were intranasally inoculated with the rSV-RSV-F vaccine, or wild-type SV. In some experiments, animals also received a previously-described rSV-RSV-G vaccine [29; 31] or an equal mixture of the rSV-RSV-F and rSV-RSV-G constructs. In each experiment, the total inoculum was 2×10^6 PFU/animal. Animals were rested for at least 9–10 days, after which mediastinal lymph nodes were collected for T-cell assays. Animals were rested for at least one month post-vaccination prior to serum sample collections and challenges.

Enzyme-linked immunosorbent assay (ELISA)

For detection of anti-RSV F-specific antibodies, ELISA plates (96-well) were coated with 1 µg/ml purified F protein (see below) and incubated overnight. Plates were blocked with PBS/3% bovine serum albumin (BSA, Sigma, St Louis, MO), after which serially diluted serum samples from the test and control cotton rats were added and incubated for at least 2 hrs at 37 °C. Plates were then washed and incubated with rabbit anti-cotton rat IgG (Virion Systems, Rockville, MD) for 30 min at room temperature. Plates were then washed, incubated with an anti-rabbit IgG-horseradish peroxidase conjugate (diluted 1:3,000 in PBS/1% BSA, Bio-Rad, Hercules, CA, Cat# 170–6515) for 30 min at room temperature, washed again, and incubated with 2,2'-azino-bis-(3-ethylbenzthiazolinesulfonic acid) (ABTS, Southern Biotechnology Associates, Inc, Birmingham, AL). Optical readings were at 405 nm.

To isolate purified RSV F protein, we utilized a recombinant SV construct that was designed to express a truncated, rather than a full-length F molecule. This SV was grown in embryonated hens eggs. Allantoic fluids were harvested three days later and fluids were centrifuged (approximately 300 × g, 10 min, to remove debris) and filtered (0.45 µm filter). Samples were passed over a PBS-equilibrated Sepharose column bound by the anti-F monoclonal antibody Palivizumab (Synagis; MedImmune Inc., Gaithersburg, MD). The column was washed with PBS and eluted with 0.2 M glycine (pH 2.8), after which pooled protein fractions were dialyzed overnight against PBS. RSV F from RSV-infected cells could also be used as the target antigen in ELISAs.

Neutralization assays and challenges

Neutralization tests were with viruses from RSV A and B isolates. Isolates were A2 (subtype A), B1 (subtype B) and VR1580 (subtype B, from the American Type Culture Collection, ATCC, Rockville, MD) and K1013 (subtype A), K1014 (subtype A), K1015 (subtype A), and K1036 (subtype B, kindly provided by Dr. J. Devincenzo, LeBonheur Children's Hospital, Memphis, TN). Each virus was grown on HEp-2 cells except for RSV B1, which was grown on Vero cells. To conduct neutralization assays, serially diluted sera were mixed with RSV (100 to 150 PFU/well) in EMEM (Cambrex Bio Science Walkersville, Inc, Walkersville, MD) for 1 hr at 37°C. The virus-serum mixtures were inoculated onto cell monolayers in 12-well plates. Plates were incubated for 1 hr (37°C, 5% CO₂) and then overlaid with medium supplemented with glutamine, antibiotics, 10% fetal calf serum and 0.75% methylcellulose (Fisher Scientific, Fair Lawn, NJ). After incubation for 4 to 6 days (37°C, 5% CO₂), the methylcellulose was removed, cells were fixed with 1 ml of formalin phosphate (1:10 dilution; PROTOCOL® from Fisher Scientific) and stained with hematoxylin and eosin. Plaques were counted and the plaque numbers were compared to those in wells with normal cotton rat sera.

Animals were challenged intranasally with RSV isolates A2, B1, K1015 or VR1580 at doses of $1.5\text{--}7.5 \times 10^6$ PFU/cotton rat. Lungs were harvested 3 days later for virus measurements and 5 days later for immunopathology studies (as described by Prince et. al. [5]).

Peptide synthesis and IFN-gamma ELISPOT assays

For analyses of RSV-specific T-cells, overlapping peptides (derived from the RSV A2 F sequence, Genbank Accession # AAB86664) were prepared by the Hartwell Center for Bioinformatics and Biotechnology at St. Jude Children's Research Hospital. Peptides were generally 15 amino acids in length and were initiated at intervals of 5 amino acids to cover the entire length of the RSV F protein. They were then combined to form eleven pools (see detail below).

Multiscreen-hemagglutinin filtration plates (Millipore, Bedford, MA) were incubated overnight at 4°C with 3.3 µg/ml goat anti-cotton rat IFN-gamma antibody (R & D Systems, Minneapolis, MN), washed, and incubated for at least 1 hr at 37°C with complete tumor medium (CTM [32;33], a modified Eagle's medium [Invitrogen, Grand Island, NY] supplemented with 10% fetal calf serum, dextrose [500 µg/ml], glutamine [2 mM], 2-mercaptoethanol [3×10^{-5} M], essential and non-essential amino acids, sodium pyruvate, sodium bicarbonate, and antibiotics). Mediastinal lymph node cells were harvested from cotton rats 10 days after vaccination. Fresh cells were suspended in CTM and added to wells ($2.5\text{--}1 \times 10^6$ cells/well) containing the peptide pools (final concentration of each peptide, approximately 10 µM). Positive control wells received 4 µg/ml Con A (Sigma-Aldrich, St. Louis, MO) rather than peptide. The plates were incubated for 48 hr at 37 °C, washed four times with PBS, and washed four times with PBS wash buffer (PBS with 0.05% Tween 20). Biotinylated goat anti-cotton rat IFN-gamma antibody (100 µl of 0.5 µg/ml; R & D Systems, Minneapolis, MN) in PBS containing 0.05% Tween 20 and 1% FCS was then added to each well, and plates were incubated at 37 °C for at least 2 hr. After additional washing, streptavidin-conjugated alkaline phosphatase (Cat# D0396, DAKO, Copenhagen, Denmark) diluted 1:500 in PBS wash buffer was added. One hour later, plates were rinsed with wash buffer and water and the IFN-gamma spots were developed by adding 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium alkaline phosphatase substrate (Sigma-Aldrich). Spots were counted with an Axioplan 2 microscope and software (Carl Zeiss, Munich-Hallbergmoos, Germany).

Measurement of virus replication

Three days after intranasal RSV challenge, virus was quantified in the lungs of vaccinated animals. Briefly, cotton rats were sacrificed and the lungs were harvested and homogenized on ice with a mechanical Dounce homogenizer (PowerGen125 PCR Tissue Homogenizing kit; Fisher Scientific) to yield a final homogenate of 5 ml in PBS. Homogenates were centrifuged (approximately $1500 \times g$, 10 min) and supernatants were collected for cryopreservation prior to testing. For all viruses except the B1 isolate, serially diluted supernatants were inoculated into multiple wells of HEP-2 cell monolayers in 6–12 well plates in EMEM; after 1 hr at 37 °C, 5% CO₂, the wells were overlaid with medium supplemented with glutamine, antibiotics, 10% fetal calf serum and 0.75% methylcellulose. After incubation for 4 to 6 days (37°C, 5% CO₂), the methylcellulose was removed, the cells were fixed with formalin phosphate, and the plates were stained with hematoxylin and eosin for plaque counting. For RSV B1, the same procedure was followed except that samples were applied to Vero cell monolayers (ATCC, Rockville, MD). Plaque forming units within cotton rat lungs were calculated.

Histopathology studies

Lungs isolated 5 days after challenge with RSV were prepared for histological analyses as previously described [29]. Briefly, lungs were inflated via the trachea with 10% neutral buffered formalin and submerged in formalin for overnight fixation. The fixed tissue was

embedded in paraffin, sectioned at 4 μ m, and stained with hematoxylin and eosin. Lung pathology was scored on the basis of three parameters: peribronchiolitis (inflammatory cells around small airways); alveolitis (inflammatory cells within alveolar spaces); and interstitial pneumonitis (inflammatory cell infiltrates and thickening of alveolar walls). Scoring was performed as described by Prince et. al. [5] in a blinded manner by a veterinary pathologist (KB) using a scale of 0 (no inflammation) to 100 (maximum inflammation within the experiment). Relative comparisons between test and control animals were evaluated independently for each parameter and for each experiment.

RESULTS

SV recombinant construction and assessment

Recombinant rSV-RSV-F particles were constructed to carry the F gene of RSV (see Figure 1A and Materials and Methods section for construction details). Gel electrophoresis/Western blot was then used to test for F expression among cells infected with the recombinant virus. RSV F proteins were expressed by cells infected with the construct and were predictably the same size as the F protein from RSV-infected cells (data not shown).

We questioned whether the RSV F protein might be present on the recombinant SV virus particle. Our expectations were that SV virions would not contain the foreign RSV F protein, because it lacked cytoplasmic sequences required for incorporation into SV virions [34]. To confirm this expectation, rSV-RSV-F virus particles were purified from HEp 2 infected cells and were tested by gel electrophoresis and Western blotting. In Figure 1B (top panel) are shown the results of gels loaded with RSV-infected cell lysates (lanes 1–3) and purified rSV-RSV-F virus particles (lanes 4–6), stained with GelCode Blue. The pattern of recombinant SV (lanes 4–6) typified that of unmodified SV, in that external (e.g. HN_d) and internal (e.g. NP) proteins were clearly resolved. In Figure 1B (bottom panel) are shown the same samples resolved by SDS-PAGE and subjected to Western blotting with an anti-RSV F monoclonal antibody. Despite clear resolution of F in the control RSV lysates (lanes 1–3), there was no evidence of RSV F within the SV recombinant virus particles. These findings confirmed that the RSV F protein was expressed following cell infection, but was not incorporated into the membrane of SV particles.

rSV-RSV-F elicits RSV-specific binding and neutralizing antibodies in a cotton rat model

To determine whether the RSV F protein was immunogenic in the context of an rSV-RSV-F infection, we inoculated groups of 5 cotton rats intranasally with the recombinant virus (2×10^6 PFU/rat). Blood was collected four weeks after inoculation for antibody studies by ELISA using purified RSV F protein as the target antigen. The testing of pooled, serially diluted sera (1:50, 1:500 and 1:5000) from rSV-RSV-F immunized cotton rats revealed significant levels of anti-RSV F antibodies, whereas sera from control cotton rats (immunized with unmodified SV) were negative (Figure 2A). To assess the durability of the antibody response, we tested one group of animals by ELISA 8 to 12 months after immunization with rSV-RSV-F. Antibody responses were detected at this time (1:5,000 dilution, data not shown). This result was consistent with previous studies demonstrating the longevity of immune responses triggered by live viral vaccines [35;36]. Our general experience with recombinant SV (expressing passenger genes from RSV or other sources) was that the viruses could be transiently detected in the cotton rat lungs for up to 5 days after infection with no signs of weight loss or disease. Possibly, the limited virus growth without disease contributed to the generation of robust and durable immune responses.

Given that RSV-specific antibodies were evident in vaccinated cotton rats, we next questioned whether RSV neutralizing activities could be measured. Pooled serially diluted serum samples

obtained four weeks after inoculation of cotton rats with rSV-RSV-F were incubated with RSV-A2 and plaque assays were conducted. These sera were highly efficient at RSV neutralization (Figure 2B). Control sera collected before inoculation (not shown) or from control unmodified SV-inoculated animals showed no effect. These results were consistent among replicate experiments and revealed that high-titered binding and neutralizing antibody activities could be demonstrated in rSV-RSV-F-immunized animals.

rSV-RSV-F elicits RSV-specific T-cell responses

Intranasal SV is well known for its capacity to elicit both B- and T-cell responses within the lung and local draining lymph nodes [37]. Accordingly, we conducted IFN- γ -ELISPOT assays to identify vaccine-induced, RSV-specific T cell responses. Mediastinal lymph nodes (MLN) were harvested from cotton rats 10 days after intranasal inoculation with rSV-RSV-F or a wild-type SV control. To examine IFN- γ expression, we incubated MLN cell suspensions with 11 pools of overlapping peptides representing the entire RSV F protein (see Figure 3 for peptide locations). As shown in Figure 4, rSV-RSV-F successfully induced IFN- γ -producing T cell responses, targeted primarily toward epitopes in the N-terminal and mid-region of the F protein.

No enhanced pathology in the lungs of vaccinated animals after challenge with RSV

We next questioned whether vaccination with rSV-RSV-F vaccine might exacerbate disease following a challenge with RSV A2. We therefore rested vaccinated and control animals for 5 weeks and then challenged animals intranasally with RSV-A2 (1.5×10^6 PFU/cotton rat). After 5 days, histopathological analyses of the lungs were performed. This test design was based on a system previously developed by Prince et. al. for the analysis of RSV-induced lung immunopathology in a cotton rat model [5]. The lungs were harvested, perfused with formalin, sectioned, and stained with hematoxylin and eosin for histologic examination. Lung pathology (peribronchiolitis, interstitial pneumonitis, and alveolitis) was scored in a blinded fashion according to previously-published criteria. There was no significant difference between immunopathology scores in rSV-RSV-F-primed or SV-primed animals compared to PBS controls, demonstrating that immunopathology was not enhanced by vaccination in this model (Table 1 shows mean scores, Figure 5 shows representative results). Of note, our previous study of rSV-RSV-G or SV-primed cotton rats also showed no enhanced immunopathology compared to PBS controls [29;31].

rSV-RSV-F vaccine confers protection against RSV challenge in the cotton rat model

Having identified the production of binding and neutralizing antibodies and T cell responses toward RSV, and having shown no enhanced immunopathology upon challenge, we next assessed the capacity of the vaccine to protect animals from the RSV challenge. As before, vaccinated (rSV-RSV-F) and control (unmodified SV or PBS) animals were rested for 5 weeks and then challenged intranasally with RSV-A2 (1.5×10^6 PFU/cotton rat). Three days after challenge, animals were sacrificed and lungs were collected for determination of RSV burden. As shown in Figure 6A, animals vaccinated with a single dose of the recombinant SV vaccine were protected from RSV challenge: no infectious RSV was isolated from their lungs. In contrast, all animals inoculated with wild-type SV and subsequently challenged with RSV showed significant virus infection. This protection illustrates the remarkable capacity of the SV recombinant to prevent infections with RSV.

The combination and testing of SV recombinants expressing RSV F and G membrane proteins

The above-described experiments clearly demonstrated the efficacy of the rSV-RSV-F construct as a vaccine for RSV. Results were, in fact, reminiscent of our previous tests with an SV recombinant expressing the RSV G protein [29;31]. In each case, constructs elicited robust

immune activities and protection. We therefore questioned whether a combination of the two vaccines would be superior to the use of either one alone. Cotton rats were inoculated with a total of 2×10^6 pfu SV by intranasal inoculation, with individual rSV-RSV-F and rSV-RSV-G vaccines or with a mixture of the two. The animals were then challenged with a higher than normal dose of RSV (7.5×10^6 PFU/cotton rat) to override the complete protection otherwise conferred by the individual rSV-RSV-F or rSV-RSV-G vaccines. As shown in Figure 6B, following the high dose challenge, we found that the protection afforded by the individual rSV-RSV-F vaccine was not exceeded by the rSV-RSV-G vaccine, and was not improved by mixing the two constructs together.

rSV-RSV-F elicits neutralizing antibodies toward a variety of RSV isolates inclusive of A and B subtypes

Because the RSV F protein is well conserved (and better conserved than G protein) among RSV isolates, we asked whether antibodies elicited by the rSV-RSV-F construct (derived from RSV A2) would recognize heterologous RSV isolates. We therefore tested sera from rSV-RSV-F-primed animals against four different viruses of the RSV A subtype and three different viruses of subtype B. As shown in Table 2A, sera were capable of neutralizing each of the viruses, inclusive of subtype 'A' and 'B' representatives. Neutralization activities by sera from rSV-RSV-G-primed animals [29;31] were also tested in preliminary experiments (Table 2B). In this case, the neutralization elicited by rSV-RSV-G of the 'A' subtype was slightly skewed toward 'A' viruses, but cross-neutralization activities against the 'B' isolates were again apparent. Results showed that although viruses can be categorized as 'A' and 'B' subtypes, the groups are not indicative of distinct serotypes. Rather, neutralizing responses elicited by an antigen from one subtype can recognize and neutralize viruses from another subtype. Results also highlighted the superior breadth of neutralizing activities elicited by the rSV-RSV-F vaccine.

rSV-RSV-F confers protection against RSV of subtype B

To further test the cross-reactive nature of responses elicited by rSV-RSV-F, we conducted challenges with heterologous viruses (inclusive of RSV B1, VR1580 and K1015). In each case, full protection was demonstrated (results of a VR1580 challenge are shown in Figure 7). The vaccine was clearly protective even when the challenge virus differed by origin, sequence and subtype.

DISCUSSION

Here we describe the construction of a new vaccine (rSV-RSV-F) and its testing in a cotton rat model for protection against RSV challenge. Vaccine development involved the use of reverse genetics to express RSV F full-length protein in the context of recombinant SV. We first demonstrated that F protein was expressed in cells infected with rSV-RSV-F. We also showed that despite the inclusion of the transmembrane domain in this recombinant, the F protein was not incorporated into viral particles (probably because sequences necessary for SV assembly [34] were lacking in the cytoplasmic tail of RSV F).

Tests of the recombinant vaccine in a cotton rat model demonstrated elicitation of neutralizing antibody and T cell responses. Importantly, results also showed that vaccination induced protection against experimental RSV infection in primed animals. While the contribution of B cell/T cell activity was not dissected in the current studies, it is likely that each contributed to the protective effect: antibodies provide the first line of defense by mediating virus clearance while T cells are known to secrete cytokines, kill virus-infected cells and facilitate effector functions by innate and adaptive arms of the immune system [38]. Our histological studies showed that protection was conferred in the absence of enhanced immunopathology after RSV

challenge, perhaps due to the presence of balanced neutralizing B-cell and T-cell activities [9]. Finally, we showed that the immune response was capable of responding to, and protecting against heterologous viruses, inclusive of viruses matched or mismatched with the vaccine by subtype.

The demonstration of RSV protection without enhanced lung pathology is worthy of comment, given that children vaccinated with the formalin-inactivated RSV vaccine of the 1960s experienced exacerbated disease compared to placebo controls following natural RSV infection. In fact, there were numerous hospitalizations and two deaths caused by immunizations with the formalin-inactivated vaccine [24]. The exacerbated disease was likely due to pulmonary inflammation initiated by a rigorous RSV-specific T cell response in the absence of neutralizing B cell activity. We propose that the induction of a strong neutralizing antibody response by vaccination may therefore be a crucial feature of safe vaccination. The lack of enhanced lung pathology in our study was likely because there was an immediate reduction of virus particles by the RSV-specific antibodies in the vaccinated groups. Without prolonged lung infection and tissue damage, vaccinated cotton rats may have experienced a decrease in recruitment of inflammatory cells and an inhibition of attendant cytokine release [6].

The studies described in this report also examined the effect of mixing SV-based vaccines expressing RSV F and G proteins. However, an improvement to the protective response was not revealed by mixing. The RSV F vaccine was therefore advanced as the preferred vaccine, given that the F gene is better conserved among natural RSV isolates than is G [39–42]. To determine if that conservation was supportive of cross-neutralization and cross-protection, we tested the rSV-RSV-F construct for elicitation of neutralizing and protective responses against non-homologous challenge viruses. These studies showed that cross-reactive responses were induced. Specifically, (i) a variety of isolates of subtypes A and B were effectively neutralized, and (ii) rSV-RSV-F (derived from an RSV A virus) conferred protection against challenges with RSV B. Such results show that although RSV isolates are routinely categorized as subtypes ‘A’ or ‘B’, the groupings do not define serologically distinct pathogens. Of note, the anti-RSV F monoclonal antibody Palivizumab is effective against RSV of both A and B subtypes [43; 44].

In addition to the advantages that SV provides as a naturally attenuated live virus vaccine vector (induction of durable antibody and T cell responses and protection from RSV challenge), the SV backbone itself provides protection against its human cognate, hPIV-1. Our previous studies showed that live, unmodified SV conferred protection against hPIV-1 challenge in African green monkeys. Specifically, of six monkeys vaccinated with SV, none demonstrated evidence of infectious hPIV-1 after challenge. Of six unvaccinated monkeys, all became infected [25].

The use of unmodified SV as a human vaccine for hPIV-1 is now being studied in clinical trials. No serious or unexpected adverse events have been observed [26]. Recent research by Bousse et. al. [45] provides at least one explanation for this finding. These researchers compared the growth of human (hPIV-1) and mouse (SV) parainfluenza viruses on human respiratory cells in vitro and found that unlike hPIV-1, SV was naturally attenuated in the context of human interferon-mediated protective mechanisms. This may explain the lack of SV association with known human diseases, despite abundant contact between humans and mice (the natural hosts of SV). An intranasal paramyxovirus vaccine obviously holds great clinical appeal, particularly because it can be administered to the target pediatric population (12 months of age [1]) without needle sticks. Another practical advantage of SV is that it grows to high titers in hens eggs, facilitating the manufacture of large vaccine lots.

In conclusion, we have demonstrated that a new SV-based RSV F construct is capable of conferring protection against RSV in a cotton rat model. We propose that the rSV-RSV-F recombinant may ultimately be found to serve a dual purpose: the xenogenic backbone may protect children from hPIV-1 while the passenger gene may simultaneously protect children from RSV. Obviously, the prevention of these two severe respiratory diseases with a single intranasal administration would constitute an enormous advance and would significantly decrease morbidity and mortality in the human population.

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References

1. McCormick J, Tubman R. Readmission with respiratory syncytial virus (RSV) infection among graduates from a neonatal intensive care unit. *Pediatr Pulmonol* 2002 Oct;34(4):262–6. [PubMed: 12205567]
2. Collins, PL.; Chanock, RM.; Murphy, BR. Respiratory Syncytial Virus. In: Knipe, DM.; Howley, PM., editors. *Fields Virology*. 4. Philadelphia, PA: Lippincott Williams and Wilkins; 2001. p. 1443-85.
3. Anderson LJ, Parker RA, Strikas RL. Association between respiratory syncytial virus outbreaks and lower respiratory tract deaths of infants and young children. *J Infect Dis* 1990 Apr;161(4):640–6. [PubMed: 2319164]
4. Fulginiti VA, Eller JJ, Sieber OF, Joyner JW, Minamitani M, Meiklejohn G. Respiratory virus immunization I. A field trial of two inactivated respiratory virus vaccines; an aqueous trivalent parainfluenza virus vaccine and an alum-precipitated respiratory syncytial virus vaccine. *Am J Epidemiol* 1969;89:435–48. [PubMed: 4305199]
5. Prince GA, Curtis SJ, Yim KC, Porter DD. Vaccine-enhanced respiratory syncytial virus disease in cotton rats following immunization with Lot 100 or a newly prepared reference vaccine. *J Gen Virol* 2001 Dec;82(Pt 12):2881–8. [PubMed: 11714962]
6. Johnson TR, Graham BS. Secreted respiratory syncytial virus G glycoprotein induces interleukin-5 (IL-5), IL-13, and eosinophilia by an IL-4-independent mechanism. *J Virol* 1999 Oct;73(10):8485–95. [PubMed: 10482601]
7. Johnson TR, Johnson JE, Roberts SR, Wertz GW, Parker RA, Graham BS. Priming with secreted glycoprotein G of respiratory syncytial virus (RSV) augments interleukin-5 production and tissue eosinophilia after RSV challenge. *J Virol* 1998 Apr;72(4):2871–80. [PubMed: 9525607]
8. Murphy BR, Prince GA, Walsh EE, et al. Dissociation between serum neutralizing and glycoprotein antibody responses of infants and children who received inactivated respiratory syncytial virus vaccine. *J Clin Microbiol* 1986 Aug;24(2):197–202. [PubMed: 3755730]
9. Srikiatkachorn A, Braciale TJ. Virus-specific CD8+ T lymphocytes downregulate T helper cell type 2 cytokine secretion and pulmonary eosinophilia during experimental murine respiratory syncytial virus infection. *J Exp Med* 1997 Aug 4;186(3):421–32. [PubMed: 9236194]
10. Sparer TE, Matthews S, Hussell T, et al. Eliminating a region of respiratory syncytial virus attachment protein allows induction of protective immunity without vaccine-enhanced lung eosinophilia. *J Exp Med* 1998 Jun 1;187(11):1921–6. [PubMed: 9607931]
11. Tebbey PW, Hagen M, Hancock GE. Atypical pulmonary eosinophilia is mediated by a specific amino acid sequence of the attachment (G) protein of respiratory syncytial virus. *J Exp Med* 1998 Nov 16;188(10):1967–72. [PubMed: 9815273]
12. Afghani B, Ngo T, Leu SY, et al. The effect of an interventional program on adherence to the american academy of pediatrics guidelines for palivizumab prophylaxis. *Pediatr Infect Dis J* 2006 Nov;25(11):1019–24. [PubMed: 17072124]

13. Mitchell I, Tough S, Gillis L, Majaesic C. Beyond randomized controlled trials: a “real life” experience of respiratory syncytial virus infection prevention in infancy with and without palivizumab. *Pediatr Pulmonol* 2006 Dec;41(12):1167–74. [PubMed: 17058279]
14. Kusuda S, Koizumi T, Sakai T, Fujimura M, Nishida H, Togari H. Results of clinical surveillance during the Japanese first palivizumab season in 2002–2003. *Pediatr Int* 2006 Aug;48(4):362–8. [PubMed: 16911080]
15. Buchholz UJ, Granzow H, Schuldt K, Whitehead SS, Murphy BR, Collins PL. Chimeric bovine respiratory syncytial virus with glycoprotein gene substitutions from human respiratory syncytial virus (HRSV): effects on host range and evaluation as a live-attenuated HRSV vaccine. *J Virol* 2000 Feb;74(3):1187–99. [PubMed: 10627529]
16. Schmidt AC, McAuliffe JM, Murphy BR, Collins PL. Recombinant bovine/human parainfluenza virus type 3 (B/HPIV3) expressing the respiratory syncytial virus (RSV) G and F proteins can be used to achieve simultaneous mucosal immunization against RSV and HPIV3. *J Virol* 2001 May;75(10):4594–603. [PubMed: 11312329]
17. Crowe JE Jr, Bui PT, Siber GR, Elkins WR, Chanock RM, Murphy BR. Cold-passaged, temperature-sensitive mutants of human respiratory syncytial virus (RSV) are highly attenuated, immunogenic, and protective in seronegative chimpanzees, even when RSV antibodies are infused shortly before immunization. *Vaccine* 1995 Jun;13(9):847–55. [PubMed: 7483808]
18. Murphy BR, Hall SL, Kulkarni AB, et al. An update on approaches to the development of respiratory syncytial virus (RSV) and parainfluenza virus type 3 (PIV3) vaccines. *Virus Res* 1994 Apr;32(1):13–36. [PubMed: 8030364]
19. Zeng RH, Gong W, Fan CF, Wang YF, Mei XG. Induction of balanced immunity in BALB/c mice by vaccination with a recombinant fusion protein containing a respiratory syncytial virus G protein fragment and a CTL epitope. *Vaccine* 2006 Feb 13;24(7):941–7. [PubMed: 16159685]
20. Yusibov V, Mett V, Mett V, et al. Peptide-based candidate vaccine against respiratory syncytial virus. *Vaccine* 2005 Mar 18;23(12):2261–5.
21. Elliott MB, Pryharski KS, Yu Q, et al. Recombinant respiratory syncytial viruses lacking the C-terminal third of the attachment (G) protein are immunogenic and attenuated in vivo and in vitro. *J Virol* 2004 Jun;78(11):5773–83. [PubMed: 15140975]
22. Power UF, Plotnicky H, Blaেকে A, Nguyen TN. The immunogenicity, protective efficacy and safety of BBG2Na, a subunit respiratory syncytial virus (RSV) vaccine candidate, against RSV-B. *Vaccine* 2003 Dec 12;22(2):168–76. [PubMed: 14615143]
23. Tang RS, Schickli JH, MacPhail M, et al. Effects of human metapneumovirus and respiratory syncytial virus antigen insertion in two 3′ proximal genome positions of bovine/human parainfluenza virus type 3 on virus replication and immunogenicity. *J Virol* 2003 Oct;77(20):10819–28. [PubMed: 14512532]
24. Girard MP, Cherian T, Pervikov Y, Kieny MP. A review of vaccine research and development: human acute respiratory infections. *Vaccine* 2005 Dec 30;23(50):5708–24. [PubMed: 16154667]
25. Hurwitz JL, Soike KF, Sangster MY, et al. Intranasal Sendai virus vaccine protects African green monkeys from infection with human parainfluenza virus-type one. *Vaccine* 1997;15:533–40. [PubMed: 9160521]
26. Slobod KS, Shenep JL, Lujan-Zilbermann J, et al. Safety and immunogenicity of intranasal murine parainfluenza virus type 1 (Sendai virus) in healthy human adults. *Vaccine* Aug 13;2004;22, 23–24, 3182–6.
27. Bousse T, Matrosovich T, Portner A, Kato A, Nagai Y, Takimoto T. The long noncoding region of the human parainfluenza virus type 1 f gene contributes to the read-through transcription at the m-f gene junction. *J Virol* 2002 Aug;76(16):8244–51. [PubMed: 12134030]
28. Kato A, Sakai Y, Shioda T, Kondo T, Nakanishi M, Nagai Y. Initiation of Sendai virus multiplication from transfected cDNA or RNA with negative or positive sense. *Genes Cells* 1996 Jun;1(6):569–79. [PubMed: 9078386]
29. Takimoto T, Hurwitz JL, Coleclough C, et al. Recombinant Sendai virus expressing the G glycoprotein of respiratory syncytial virus (RSV) elicits immune protection against RSV. *J Virol* 2004 Jun;78(11):6043–7. [PubMed: 15141002]

30. Beeler JA, van Wyke CK. Neutralization epitopes of the F glycoprotein of respiratory syncytial virus: effect of mutation upon fusion function. *J Virol* 1989 Jul;63(7):2941–50. [PubMed: 2470922]
31. Takimoto T, Hurwitz JL, Zhan X, et al. Recombinant Sendai virus as a novel vaccine candidate for respiratory syncytial virus. *Viral Immunol* 2005;18(2):255–66. [PubMed: 16035938]
32. Woodland DL, Happ MP, Bill J, Palmer E. Requirement for cotolerogenic gene products in the clonal deletion of I-E reactive cells. *Science* 1990;247:964–7. [PubMed: 1968289]
33. Kappler JW, Skidmore B, White J, Marrack P. Antigen-inducible, H-2-restricted, interleukin-2-producing T cell hybridomas. Lack of independent antigen and H-2 recognition. *J Exp Med* 1981 May 1;153(5):1198–214. [PubMed: 6166712]
34. Takimoto T, Bousse T, Coronel EC, Scroggs RA, Portner A. Cytoplasmic domain of Sendai virus HN protein contains a specific sequence required for its incorporation into virions. *J Virol* 1998 Dec; 72(12):9747–54. [PubMed: 9811709]
35. Amanna IJ, Slifka MK, Crotty S. Immunity and immunological memory following smallpox vaccination. *Immunol Rev* 2006 Jun;211, 320–37.
36. Crotty S, Felgner P, Davies H, Glidewell J, Villarreal L, Ahmed R. Cutting edge: long-term B cell memory in humans after smallpox vaccination. *J Immunol* 2003 Nov 15;171(10):4969–73. [PubMed: 14607890]
37. Sangster M, Hyland L, Sealy R, Coleclough C. Distinctive kinetics of the antibody-forming cell response to Sendai virus infection of mice in different anatomical compartments. *Virology* 1995;207:287–91. [PubMed: 7871740]
38. Janeway, CA., Jr; Travers, P.; Walport, M.; Shlomchik, M. Immunobiology, the immune system in health and disease. 6. New York, NY: Garland Publishing; 2005.
39. Parveen S, Sullender WM, Fowler K, Lefkowitz EJ, Kapoor SK, Broor S. Genetic variability in the G protein gene of group A and B respiratory syncytial viruses from India. *J Clin Microbiol* 2006 Sep; 44(9):3055–64. [PubMed: 16954227]
40. Cane PA, Matthews DA, Pringle CR. Frequent polymerase errors observed in a restricted area of clones derived from the attachment (G) protein gene of respiratory syncytial virus. *J Virol* 1993 Feb; 67(2):1090–3. [PubMed: 7678299]
41. Lim CS, Kumarasinghe G, Chow VT. Sequence and phylogenetic analysis of SH, G, and F genes and proteins of Human respiratory syncytial virus isolates from Singapore. *Acta Virol* 2003;47(2):97–104. [PubMed: 14524476]
42. Venter M, Madhi SA, Tiemessen CT, Schoub BD. Genetic diversity and molecular epidemiology of respiratory syncytial virus over four consecutive seasons in South Africa: identification of new subgroup A and B genotypes. *J Gen Virol* 2001 Sep;82(Pt 9):2117–24. [PubMed: 11514720]
43. Saez-Llorens X, Moreno MT, Ramilo O, Sanchez PJ, Top FH Jr, Connor EM. Safety and pharmacokinetics of palivizumab therapy in children hospitalized with respiratory syncytial virus infection. *Pediatr Infect Dis J* 2004 Aug;23(8):707–12. [PubMed: 15295219]
44. Simoes EA, Groothuis JR, Carbonell-Estrany X, et al. Palivizumab prophylaxis, respiratory syncytial virus, and subsequent recurrent wheezing. *J Pediatr* 2007 Jul;151(1):34–42. [PubMed: 17586188]
45. Bousse T, Chambers RL, Scroggs RA, Portner A, Takimoto T. Human parainfluenza virus type 1 but not Sendai virus replicates in human respiratory cells despite IFN treatment. *Virus Res* 2006 Oct;121 (1):23–32. [PubMed: 16677733]

A. pSV-RSV-F **B.** **Figure 1. Design and characterization of recombinant Sendai virus expressing the RSV F protein**

A. A schematic representation is shown of the pSV-RSV-F clone used to produce the recombinant virus. A unique *NotI* restriction enzyme site was created in the non-coding region of the HN gene of the full genome SV cDNA for insertion of the RSV F gene. The RSV gene was captured by RT-PCR, digested with *NotI* and cloned into the *NotI* site of pSV(+)N or pSV(E). T7=T7 promoter; ribo=hepatitis delta virus ribozyme sequence.

B. To examine F expression on rSV-RSV-F virions, SDS gels were prepared with three dilutions of RSV-infected HEp-2 cell lysates (positive controls, 4, 2 and 1 µg, left 3 lanes) and three dilutions of rSV-RSV-F virions purified from infected HEp-2 cells (12, 8, and 4 µg; right 3 lanes). The locations of SV proteins on GelCode Blue-stained gels are indicated: L-large protein, HNnd-hemagglutinin-neuraminidase dimer, P-polymerase, F₀-uncleaved fusion protein, NP-nucleoprotein, M- matrix protein (upper panel). Proteins were transferred from SDS gels to nitrocellulose sheets for Western blot analyses with an anti-RSV F monoclonal antibody (lower panel).

A.

B.

Figure 2. Cotton rats primed with rSV-RSV-F generate RSV-specific antibodies

Groups of 5 cotton rats were inoculated with 2×10^6 PFU of rSV-RSV-F or unmodified SV. Sera were collected after 4 weeks and pooled for each group.

(A) ELISAs were conducted with serially diluted samples (from cotton rats inoculated with rSV-RSV-F or unmodified SV (control)) on plates coated with purified F protein.

(B) Pooled serum samples (from cotton rats inoculated with rSV-RSV-F or unmodified SV (control)) were tested for inhibition of RSV growth in a plaque assay. Sera were mixed with RSV-A2 for one hour and then inoculated onto HEp-2 cell monolayers. Results are the mean percent plaque reduction mediated by sera from rSV-RSV-F or SV (control)-immunized cotton rats as compared to sera from untreated animals. Standard error bars are shown. Individual sera

were also tested and each demonstrated high activity; no non-responders were evident among rSV-RSV-F-vaccinated animals.

Figure 3. Peptide pools representing the entire RSV-F sequence

The synthesized peptides were generally 15 amino acids in length and were initiated at 5-amino acid intervals to cover the entire length of the RSV F protein. Each pool comprised approximately 10 peptides. Seven peptides were not synthesized due to technical difficulties: ITTILTAVTFCFASG (pool 1); KIKSALLSTNKAVVS (pool 4); NFYDPLVFP SDEFDA (pool 10); and STTNIMITTIIIVIIIVILLSLIAVG, and VILLSLIAVGLLLYC (pool 11). The arrow indicates the position of the F transmembrane region.

Figure 4. RSV-specific T cell responses elicited by inoculation with rSV-RSV-F

Mediastinal lymph nodes (MLN) were isolated from cotton rats 10 days after inoculation with rSV-RSV-F (panel A) or unmodified SV (panel B, control) to assess T cell function by IFN- γ ELISpot activity. MLN were combined from two cotton rats (to provide enough cells for an analysis of all eleven peptide pools, x axes) and more than one cotton rat pair was tested at a time (to provide a measure of mean and standard error). Spot-forming units (SFU) were counted per well with different cell numbers per well (see legend). The number of cells aliquoted per well varied from 2.5×10^5 to 1×10^6 .

Figure 5. No enhanced lung pathology after vaccination with rSV-RSV-F and subsequent challenge with RSV

Cotton rats were inoculated with PBS, unmodified SV or rSV-RSV-F and then challenged with intranasal RSV 5 weeks later. Five days after RSV challenge, cotton rat lungs were harvested for histologic analyses. Lung analyses revealed peribronchiolitis, alveolitis, and interstitial pneumonitis (primarily composed of mononuclear cells). Representative photographs are shown for animals inoculated with PBS (left panel), SV (middle panel), or rSV-RSV-F (right panel). No enhanced inflammation was noticed among vaccinated cotton rats. Photographs of hematoxylin and eosin stain, magnification 200X.

Figure 6. Recombinant viruses confer protection against homologous RSV challenge

A. Animals inoculated with rSV-RSV-F were challenged intranasally with the homologous RSV-A2 (1.5×10^6 PFU/rat). Protection was observed relative to control animals that were immunized with unmodified SV. ND=Virus not detected. Virus replication in the lung was determined by plaque assay (see Materials and Methods). Each symbol represents an individual cotton rat.

B. Groups of cotton rats were vaccinated with rSV-RSV-F, rSV-RSV-G, a combination of the two recombinants, or unmodified SV (control). Animals were challenged with a high dose (7.5×10^6 pfu) of the homologous RSV A2 five weeks later, and virus replication was monitored in lungs after three additional days.

Figure 7.

Animals vaccinated with rSV-RSV-F or unmodified SV (control) were challenged with the RSV type B isolate, VR1580. Virus replication in the lung was determined three days later by plaque assay. Animals inoculated with rSV-RSV-F were protected against intranasal challenge (1.5×10^6 PFU/cotton rat) with the type B RSV isolate. All control animals, immunized with unmodified SV at the same dose, were infected.

Table 1
Pulmonary histopathology scores after RSV challenge

Pathology	Animal groups		
	rSV-RSV-F	Unmodified SV	PBS
Peribronchiolitis	67±10.4	90±10.0	75±26.7
Alveolitis	58±11.0	77±14.0	68±14.4
Interstitial pneumonitis	37±6.7	72±30.5	60±17.0

Legend: Cotton rats were inoculated with rSV-RSV-F, SV or PBS, challenged with RSV, and examined 5 days later. Values are the mean score $\pm$ standard deviations for five animals (0 = no inflammation; 100 = maximum inflammation scored within the experiment). The observer was blinded to sample origin during scoring.

Vaccinated cotton rat sera neutralize heterologous isolates from RSV A and B subtypes

A. Sera from rSV-RSV-F immunized cotton rats were tested for neutralization capacity against four virus isolates of RSV subtype A and three virus isolates from RSV subtype B. Neutralization was identified in every case. Sera from animals immunized with unmodified SV (SV Controls) were also tested. Percentages of neutralization were determined by comparing plaque forming units in test wells to wells containing normal cotton rat sera. Results are shown with standard errors. Activities of $\geq 30\%$ are shown in bold. Virus samples were from the American Type Tissue Culture Collection (ATCC) and Dr. J. Devincenzo (LeBonheur Children's Hospital, Memphis, TN). Percentages of neutralization, means for each subtype, and standard errors are shown. **B.** Preliminary neutralization results are shown for sera from rSV-RSV-G immunized cotton rats [29;31]. ND=Not determined.

Vaccine	Serum Dilution	Percent Neutralization Against RSV:									
		Type A Isolates					Type B Isolates				
		A2	K1013	K1014	K1015	Average	B-1	VR1580	K1036	Average	
rSV-RSV-F	1:64	86	75	91	77	82±7	100	80	72	84±12	
	1:256	54	30	87	48	55±21	81	60	50	64±13	
	1:1024	6	2	55	16	20±21	41	21	26	29±8	
SV Controls	1:64	8	10	0	7	6±4	4	5	8	6±2	
	1:256	9	2	0	19	8±7	8	0	0	3±4	
	1:1024	1	1	0	21	6±9	1	3	9	4±3	
B.											
rSV-RSV-G	1:64	53	44	77	58	58±12	30	60	14	35±19	
	1:256	48	15	71	19	38±23	28	36	10	25±11	
	1:1024	24	7	30	4	16±11	ND	5	2	4±2	