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Sendai virus recombinant vaccine expressing hPIV-3 HN or F elicits protective immunity and combines with a second recombinant to prevent hPIV-1, hPIV-3 and RSV infections

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Abstract

The human parainfluenza viruses (hPIVs) and Respiratory Syncytial Virus (RSV) are the leading causes of serious respiratory illness in the human pediatric population. Despite decades of research, there are currently no licensed vaccines for either the hPIV or RSV pathogens. Here we describe the testing of hPIV-3 and RSV candidate vaccines using Sendai virus (SeV, murine PIV-1) as a vector. SeV was selected as the vaccine backbone, because it has been shown to elicit robust and durable immune activities in animal studies, and has already advanced to human safety trials as a xenogenic vaccine for hPIV-1. Two new SeV-based hPIV-3 vaccine candidates were first generated by inserting either the fusion (F) gene or hemagglutinin-neuraminidase (HN) gene from hPIV-3 into SeV. The resultant rSeV-hPIV3-F and rSeV-hPIV3-HN vaccines expressed their inserted hPIV-3 genes upon infection. The inoculation of either vaccine into cotton rats elicited binding and neutralizing antibody activities, as well as interferon- γ -producing T-cells. Vaccination of cotton rats resulted in protection against subsequent challenges with either homologous or heterologous hPIV-3. Furthermore, vaccination of cotton rats with a mixture of rSeV-hPIV3-HN and a previously described recombinant SeV expressing the F protein of RSV resulted in protection against three different challenge viruses: hPIV-3, hPIV-1 and RSV. Results encourage the continued development of the candidate recombinant SeV vaccines to combat serious respiratory infections of children.

Keywords

respiratory syncytial virus; parainfluenza virus; protective immunity

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1. Introduction

The human parainfluenza viruses (hPIVs) and respiratory syncytial virus (RSV) are the leading causes of viral pneumonia in infants and children [1]. Among the hPIVs, the hPIV-3 subtype causes the most serious infections. In the United States, hPIV-3 epidemics occur annually during spring and summer months [1;2]. Approximately 62% of humans are infected with hPIV-3 by age 1, more than 90% by age 2, and almost 100% by age 4 [3;4].

Clinical observations have indicated that the first hPIV-3 infection is generally most severe. Re-infection with hPIV-3 occurs throughout life, but tends to result in more mild disease and is associated only infrequently with serious lower respiratory tract illness. The more mild disease is likely attributed to the larger airways of infected individuals and to the memory T-cell and B-cell activities elicited by first infections [1]. The production of an effective hPIV-3 vaccine is clearly desired as a means to combat the more serious infections of younger individuals.

Previous efforts to develop hPIV-3 vaccines have included studies of cold-adapted viruses [5-7] and bovine PIV-3 [8]. Challenges facing the advancement of cold-adapted vaccines have concerned the safety of vaccinated infants and their close contacts. In early studies, the frequency of adverse events and transmission rendered certain vaccine candidates unacceptable. However, one cold-adapted vaccine (HPIV3cp45) has met safety requirements and may continue to advance [9-11]. The main challenge facing the bovine PIV-3 strategy has been its limited antigenic relation to human PIV-3. The vaccine has appeared to be safe in humans, but has not generated protective immune responses. Researchers hope to remedy this situation by producing vaccines that recombine the hPIV-3 hemagglutinin-neuraminidase (HN) and fusion (F) genes with the bovine PIV-3 backbone [12;13].

Here, we describe a new strategy for the development of hPIV-3 vaccines: the use of reverse genetics to create Sendai virus (SeV)-based vectors that express the hPIV-3 genes HN and F. SeV (mouse PIV-1) was chosen as the delivery vehicle for these vaccines, because of its ability to prevent hPIV-1 infections in non-human primates [14;15], its natural host range restriction [16] and its safety profile in current clinical trials [16;17]. The hPIV-3 HN and F genes were selected as target antigens, because each encodes a viral membrane protein with known B-cell and T-cell immunogenicity [18-21].

In this report, we show that the SeV-based hPIV-3 vaccines not only elicit robust immune responses, but also mediate protection against homologous and heterologous hPIV-3 infections in a cotton rat model. Further, we show that a vaccine formulated by mixing one of these candidate SeV-based hPIV-3 vaccines with a previously described SeV-based RSV vaccine [22;23] protects cotton rats from challenges with three different respiratory viruses: hPIV-1, hPIV-3 and RSV.

2. Materials and Methods

2.1 Construct design

Replication-competent recombinant SeVs were rescued using a reverse genetics system, described previously [22-25]. The full-length cDNA of SeV (Enders strain) was first cloned. To this end, Enders SeV RNA was extracted from purified stock virus and reverse transcription (RT)-PCR was performed. PCR products of each gene were cloned into pTF1 and then cloned into pUC19 to construct the full genome SeV Enders cDNA (pSV(E)). The SeV genome in this clone was straddled by a T7 promoter and a hepatitis delta virus ribozyme sequence. As shown in Figure 1A, a unique *NotI* site was positioned in the non-coding region between the F and HN genes of SeV.

For cloning of the hPIV-3 F and HN genes, LLC-MK₂ cells were infected with the C243 strain of hPIV-3 (VR-93, American Type Culture Collection [ATCC], Rockland, MD) and viral RNA was extracted. The hPIV-3 F and HN genes were amplified by RT-PCR (Titan One Tube System; Roche). The PCR forward primer included a *NotI* site and the reverse primer included an SeV transcription termination signal, an intergenic (IG) sequence CTT, a transcription initiation signal, and a second *NotI* site (Figure 1, B and C). The hPIV-3 F and HN cDNAs were digested with *NotI* and ligated into the *NotI* site of pSV(E).

2.2 Virus Rescue

To rescue the recombinant viruses, we infected 293T cells with a UV-inactivated, T7 RNA polymerase-expressing recombinant vaccinia virus (vTF7.3 [26]) for 1 h at 37 °C at an MOI of 3. Cells were then co-transfected with cDNA plasmids containing either the hPIV-3 F or HN gene (1 µg) and three supporting T7-driven plasmids expressing the NP, P, or L gene of SeV (1 µg pTF1SVNP, 1 µg pTF1SVP, and 0.1 µg pTF1SVL, respectively) in the presence of Lipofectamine (Life Technologies, Grand Island, NY). Cells were then incubated for 40 h. Cell lysates were prepared and inoculated into 10-day-old embryonated chicken eggs. Allantoic fluids were harvested after 72 h and viruses were plaque purified on LLC-MK₂ cells. Recombinant SeV clones were amplified once more in embryonated eggs to prepare vaccine stocks. Recovered viruses were designated rSeV-hPIV3-F (SeV expressing hPIV-3 F protein) or rSeV-hPIV3-HN (SeV expressing hPIV-3 HN protein).

2.3 Immunoprecipitation of F and HN proteins from infected cells

To confirm that the recombinant vectors expressed hPIV-3 F or HN proteins, we examined lysates of rSeV-hPIV3-F or rSeV-hPIV3-HN infected Hep-2 cells by radio-immunoprecipitation. Briefly, Hep-2 cells were infected at an MOI of 5 with rSeV-hPIV3-F, rSeV-hPIV3-HN or wild-type SeV, and incubated at 33°C in DMEM, 10% FCS and 1% l-glutamine. Sixteen hours post-infection, the cells were washed twice with PBS containing 0.1 g/liter calcium and magnesium (PBS+). Cells were maintained in culture for 30 min in methionine- and cysteine-free medium and then labeled for 15 min with 100 µCi [³⁵S]Promix (Amersham Pharmacia Biotech) in 1 ml of DMEM lacking methionine and cysteine and containing 20 mM HEPES buffer (pH 7.3). The cells were washed once with PBS+ and chased with 3 ml of DMEM containing 2 mM methionine, 2 mM cysteine, and 20 mM HEPES buffer (pH 7.3) for 180 min. Samples were lysed with ice-cold radio-immunoprecipitation assay (RIPA) buffer containing 0.15 M NaCl, 9.25 mg/ml iodoacetamide, 1.7 mg/ml aprotinin, 10 mM phenylmethylsulfonyl fluoride. The lysate was centrifuged at 67,000 × g in an Optima TLX ultracentrifuge (Beckman Coulter). The supernatant was incubated overnight (18–22h) at 4°C with 25 µl rabbit anti-hPIV-3-F or anti-hPIV-3-HN tail peptide polyclonal antibody (1:40 dilution of rabbit sera, Harlan Bioproducts for Science, Madison WI). Immune complexes were adsorbed to protein A-Sepharose CL-4B (GE Healthcare) for 1 h at 4°C. Samples were washed three times with RIPA buffer containing 0.3 M NaCl, three times with RIPA buffer containing 0.15 M NaCl, and once with 50 mM Tris buffer (0.25 mM EDTA, 0.15 M NaCl, pH 7.4). The samples were then fractionated on 12% NuPAGE Bis-Tris polyacrylamide–SDS gels (Invitrogen). Protein bands were visualized using a Typhoon 9200 phosphorimager (GE Healthcare).

2.4 Animals, Inoculations and challenges

Groups of 5 adult female cotton rats (*Sigmodon hispidus*; Harlan Sprague Dawley, Indianapolis, IN) were intranasally inoculated with rSeV-hPIV3-F (2×10⁶ plaque-forming units [PFU]), rSeV-hPIV3-HN (2×10⁶ PFU), a mixture of 1×10⁶ rSeV-hPIV3-F and 1×10⁶ rSeV-hPIV3-HN, or a mixture of 2×10⁶ rSeV-hPIV3-HN and 2×10⁶ rSeV-RSV-F (a previously described recombinant SeV expressing the full-length RSV F protein [22]). Control

animal groups received wild-type SeV (2×10^6 PFU/cotton rat) or PBS. After 5-10 days, mediastinal lymph nodes were collected for T-cell assays. Serum samples were taken four weeks post-inoculation and challenges were performed on week five. The intranasal challenge doses were 2×10^6 PFU/cotton rat of homologous hPIV-3 (strain C243) or 1.5×10^6 PFU/cotton rat of heterologous hPIV-3 (8-94, kindly provided by Dr. R. Hayden, Clinical Virology, St. Jude Children's Research Hospital). Lungs were harvested 3 days post-challenge for virus measurements. In some experiments, animals were challenged intranasally with either hPIV-1 (C35 from ATCC, 2×10^6 PFU/cotton rat) or RSV (strain A2, 1.5×10^6 PFU/cotton rat).

2.5 Enzyme-linked immunosorbent assay (ELISA)

For studies to detect anti-hPIV-3 F- or HN-specific antibodies, an hPIV-3 stock was prepared from culture supernatants by concentration with a Millipore Amicon filter unit. Concentrates were lysed in disruption buffer (0.5% TritonX-100, 0.6M KCl, 0.05M Tris pH7.8), diluted with PBS (1:3000) and coated on 96-well ELISA plates. Lysates of wild-type SeV were plated as controls. After overnight incubation, plates were blocked with PBS containing 3% bovine serum albumin (BSA, Sigma, St Louis, MO). Serum samples from vaccinated and control animals were serially diluted and incubated on plates for 2 h at 37°C. Plates were then washed and incubated with rabbit anti-cotton rat antibody (kindly provided by Dr. Greg Prince, Virion Systems, Rockville, MD) for 30 min at room temperature. After further washing, plates were incubated with 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). Absorbance was read at 405 nm.

2.6 Neutralization assays

To conduct neutralization assays, we mixed serially diluted sera with approximately 10 TCID₅₀ hPIV-3 per well in DMEM (Cambrex Bio Science Walkersville, Inc, Walkersville, MD) for 1 h at 37°C. Viruses were either homologous (C243) or heterologous (St. Jude Children's Research Hospital isolates 4-04, 5-97 and 8-94, named by the month and year of isolation) to the vaccine. The virus-serum mixtures were then added to wells (6 wells per sample in 24-well plates) of LLC-MK₂ cell monolayers, which were incubated for 1 h (33°C, 5% CO₂) and then fed with DMEM supplemented with glutamine, antibiotics and 5% fetal calf serum (FCS). After 4 days of culture (33°C, 5% CO₂), supernatants (100 µl) from test wells were mixed with 100 µl of 0.5% fresh turkey red blood cells in round-bottomed 96-well plates and incubated at 4°C for 30 min. Hemagglutination was scored as positive or negative for each well and the percent neutralization was calculated as the reduction in frequency of positive wells for test versus control samples.

2.7 IFN-γ ELISPOT assays

For analyses of hPIV-3-specific T-cell responses, overlapping peptides (derived from the hPIV-3 F and HN sequences) 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 10 amino acids to cover the length of the hPIV-3 F and HN proteins. Pools were prepared with 10 peptides per pool for use in the ELISPOT assay.

The ELISPOT assay was conducted by incubating 3.3 µg/ml goat anti-cotton rat IFN-γ antibody (R & D Systems, Minneapolis, MN) in multiscreen-hemagglutinin filtration plates (Millipore, Bedford, MA) overnight at 4°C. After washing, the plates were blocked for at least 1 h at 37°C with complete tumor medium (CTM [27;28], a modified Eagle's medium [Invitrogen, Grand Island, NY] supplemented with 10% FCS, 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 5-10 days after vaccination. Fresh cells were suspended in CTM and added to plates at 0.25×10^6 cells per well containing individual peptide pools. The final concentration of each peptide was approximately 10 μ M. Positive control wells received 4 μ g/ml Con A (Sigma-Aldrich, St. Louis, MO) rather than peptides. The plates were incubated for 48h at 37°C and washed four times with PBS and four times with PBS wash buffer (PBS with 0.05% Tween 20). Biotinylated goat anti-cotton rat IFN- γ antibody (R & D Systems, Minneapolis, MN) was diluted in PBS (containing 0.05% Tween 20 and 1% FCS) and was added to wells (100 μ l aliquots, 0.5 μ g/ml antibody). Plates were incubated at 37°C for at least 2 h. 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. The IFN- γ 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).

2.8 Virus challenge assay

Three days after intranasal viral challenge, cotton rats were sacrificed and the lungs were harvested for measurement of virus titer. Briefly, lungs were homogenized on ice with a mechanical Dounce homogenizer (PowerGen125 PCR Tissue Homogenizing kit; Fisher Scientific) to yield 5 ml of homogenate in PBS. Homogenates were centrifuged (1500 $\times$ g, 10 min) and supernatants were collected.

For hPIV-1 detection, LLC-MK₂ cells were grown to confluency in 6-well plates in complete medium (MEM, 0.2% NaHCO₃, 2 mM glutamine, and 50 μ g/ml gentamicin) with 5% FCS. Plates were washed once with PBS/calcium/magnesium. 100 μ l of serially diluted supernatants were inoculated into wells. After 1 hr at 33°C, 5% CO₂, the cells were overlaid with 4 ml per well of complete medium supplemented with vitamins, amino acids, 0.15% BSA, 5 μ g/ml acetylated trypsin (Sigma), and 0.9% agarose (electrophoresis grade, BRL, Gaithersburg, MD). After the agarose was set, plates were inverted and incubated at 33°C in a 5% CO₂ incubator. 5 days later, plates received a second overlay (3 ml), similar to the first, but with 5% FCS instead of BSA, 0.0035% neutral red, and no trypsin supplement. Plates were incubated for one more day and plaques were counted.

For hPIV-3 detection, 100 μ l of serially diluted lung supernatants were added to wells (in 24-well plates) of LLC-MK₂ cell monolayers. Cultures were incubated for 1 hr (33°C, 5% CO₂) and then fed with DMEM supplemented with glutamine, antibiotics and 5% FCS. After 4 days incubation (33°C, 5% CO₂), 100 μ l supernatants were removed from hPIV-3-infected wells for hemagglutination assays with turkey red blood cells. TCID₅₀ were calculated using the Reed-Meunch formula.

For RSV measurements, serially diluted supernatants from lung homogenates were inoculated on Hep-2 cell monolayers in 12-well plates; after 1 h at 37 °C and 5% CO₂, the wells were overlaid with EMEM medium supplemented with glutamine, antibiotics, 10% fetal calf serum and 0.75% methylcellulose. After incubation for 5 to 6 days at 37°C and 5% CO₂, the methylcellulose was removed, cells were fixed with formalin phosphate, and the plates were stained with hematoxylin and eosin for enumeration of plaques. For each virus, the total pulmonary burden per cotton rat was scored.

3 Results

3.1 Human PIV-3 F and HN proteins are expressed by cells infected with recombinant SeV Vaccines

Recombinant SeVs were prepared by the insertion of hPIV-3 F or HN genes between the SeV F and HN genes of the full SeV Enders genome (Figure 1, panels A-C). The viruses rSeV-hPIV3-F and rSeV-hPIV3-HN were subsequently rescued and sequenced (demonstrating maintenance of passenger gene sequences). To examine expression of passenger genes by new viruses, we infected Hep-2 cells with the recombinant SeVs and performed radioimmunoprecipitation experiments. As shown in Figure 1 (panels D and E), both of the hPIV-3 proteins were expressed.

3.2 Sendai virus vaccines expressing hPIV-3 F or HN induce hPIV-3-specific binding and neutralizing antibodies in a cotton rat model

To study the immunogenicity of the SeV-based vaccines, we inoculated groups of 5 cotton rats intranasally. Each cotton rat received 2×10^6 PFU rSeV-hPIV3-F or rSeV-hPIV3-HN, or received 1×10^6 PFU of each of the two vaccines in a mixture. Unmodified SeV and PBS were used as controls. Blood was collected four weeks later for measurement of hPIV-3-specific antibodies by ELISA. Sera were pooled from each group of animals and serially diluted (1:100, 1:500 and 1:5000) for testing. All cotton rats immunized with the single or mixed recombinant vaccines showed high serum anti-hPIV-3 antibody activity (Figure 2A). Responses were not improved by use of the mixed vaccine versus rSeV-hPIV3-HN. Sera from individual cotton rats were also tested and yielded similar results (data not shown). Interestingly, cotton rats inoculated with unmodified wild-type SeV had a weak antibody reaction toward hPIV-3. This cross-reactive response was not surprising as PIV-1 and PIV-3, both respiroviruses, have sequence and antigenic similarities [19;29;30].

Sera from all groups of cotton rats were also tested for antibody responses to the SeV backbone by ELISA with SeV lysate as the target antigen (Figure 2B). SeV-specific antibody activity was induced by both the recombinant and wild-type viruses.

Having identified PIV-3-specific antibodies, we next investigated neutralizing activity. Serum samples taken four weeks after inoculation were highly efficient at neutralizing the homologous C243 hPIV-3 isolate in tissue culture (Table 1, column 3). Results showed that the rSeV-hPIV3-HN vaccine elicited higher responses than the rSeV-hPIV3-F vaccine. In the former case, neutralization was evident at serum dilutions of $>1,000$ (neutralizing activity was reduced at a serum dilution of 1: 4096, data not shown). Again, the mixed vaccine was not superior to rSeV-hPIV3-HN. We also observed that sera from animals immunized with wild-type SeV were able to neutralize the infectious hPIV-3, but this activity was only evident at a serum dilution of 1:16.

To characterize further the vaccines, we tested neutralizing activities toward heterologous hPIV-3 isolates. For these tests, we used viruses that had been isolated from several different yearly outbreaks of hPIV-3 (viruses 4-04, 5-97 and 8-94 were obtained from the Clinical Pathology Department of St. Jude Children's Research Hospital). As shown in Table 1 (columns 4-6), all of these viruses were neutralized by sera from vaccinated animals. Again, sera from the vaccine expressing the HN gene showed the best neutralization results (positive scores were evident at serum dilutions $>1,000$). The mixed vaccine did not enhance neutralizing antibody activity compared to rSeV-PIV3-HN. The results with heterologous viruses demonstrated the potent cross-neutralizing capability of antibodies elicited by recombinant SeV hPIV-3 vaccines.

3.3 Both rSeV-hPIV3-F and rSeV-hPIV3-HN elicit hPIV3-specific T-cell responses

Intranasal inoculation with SeV is well known for its capacity to elicit B- and T-cell responses within the lung and local draining lymph nodes [14;20;31-33]. Accordingly, we conducted IFN- γ -ELISPOT assays to identify vaccine-induced, hPIV-3-specific T-cell responses. For this study, mediastinal lymph nodes (MLN) were harvested from cotton rats 5-10 days after intranasal inoculation with rSeV-hPIV3-F, rSeV-hPIV3-HN or wild-type SeV. T-cells were then isolated from the draining MLN for testing against pooled peptides representing HN and F proteins (see Figure 3, panels A and B, for peptide locations). Data showed that both rSeV-hPIV3-F and rSeV-hPIV3-HN recombinants induced virus-specific T-cells able to produce IFN- γ (Figure 3, panels C and D). The MLN from cotton rats that had received only unmodified SeV were also tested for T-cell responses toward the hPIV-3 F and HN proteins (Figure 3, panels E and F). In SeV-primed animals, a significant response was demonstrated toward hPIV-3 F, reflecting sequence similarities between the F proteins of SeV and hPIV-3 [30].

3.4 rSeV-hPIV3-F and rSeV-hPIV3-HN vaccines confer complete protection against homologous and heterologous hPIV-3 challenges in the cotton rat model

Having identified the induction of binding and neutralizing antibodies and T-cell responses toward hPIV-3, we next assessed protection from hPIV-3 challenge. Five weeks after inoculations with rSeV-hPIV3-F, rSeV-hPIV3-HN, a mixture of the two constructs, wild-type SeV or PBS, cotton rats were challenged intranasally with the homologous hPIV-3 C243 strain at a dose of 2×10^6 PFU/animal. Three days after challenge, animals were sacrificed and lungs were collected for determination of hPIV-3 burden. As shown in Figure 4A, animals vaccinated with either or both of the recombinant SeV vaccines were completely protected from hPIV-3 challenge. Wild-type SeV conferred partial protection as compared to the PBS inoculation. The results clearly demonstrated the efficacy of the rSeV-hPIV3-F and rSeV-hPIV3-HN constructs as vaccines against hPIV-3.

To investigate further the protective capacities of the hPIV-3 constructs, we challenged vaccinated animals with a heterologous hPIV-3 isolate. Again, experiments were performed with groups of animals (five cotton rats per group) immunized with rSeV-hPIV3-F or rSeV-hPIV3-HN either independently or in combination. As shown in Figure 4B, all of the recombinant vaccines provided protection.

3.5 A mixed rSeV-RSV-F and rSeV-hPIV3-HN vaccine confers protection against RSV, hPIV-3 and hPIV-1 challenges in the cotton rat model

Finally, we tested whether a mixture of vaccines could be used to protect against more than one virus pathogen. Our previous work showed that the unmodified SeV protected against hPIV-1 [14] and that recombinants expressing either RSV G or F protected against RSV [22; 23;25]. To determine whether a mixture of vaccines could be used to protect against hPIV-1, hPIV-3 and RSV, we combined two constructs expressing RSV and hPIV-3 antigens in a single vaccine formulation. Specifically, we chose to combine the rSeV-RSV-F and rSeV-hPIV3-HN viruses. The rSeV-RSV-F vector was chosen rather than rSeV-RSV-G in this study, because of the greater conservation of RSV F sequences in nature [34] and because RSV G has been reported to be associated with enhanced inflammation and eosinophilia in some mouse studies [35-37]). The rSeV-PIV3-HN construct was chosen over rSeV-PIV3-F because of the greater antibody neutralization observed following rSeV-hPIV3-HN immunizations (Table 1).

Recombinants were mixed so that each virus was included at a dose of 2×10^6 PFU per inoculation. Groups of cotton rats then received the mixed vaccine, the wild-type SeV or PBS. Five weeks after vaccination, the groups were challenged with RSV, hPIV-3 or hPIV-1. The animals were sacrificed three days later for lung harvest and virus titration. As shown in Figure 5 (panels A-C), cotton rats inoculated with the mixed vaccine were successfully protected

against all three pathogens. The wild-type SeV also conferred protection against hPIV-1 and partial protection against hPIV-3. Together, these findings demonstrated that inoculation with a mixture of two different recombinant SeVs was sufficient to prevent infection with three different viruses in the cotton rat model.

4. Discussion

This report describes two new recombinant SeV vaccines that express the hPIV-3 F (rSeV-hPIV3-F) and HN (rSeV-hPIV3-HN) proteins, respectively. We initiated studies by demonstrating PIV-3 protein expression by cells infected with the recombinant SeVs. We then employed a cotton rat model to show that each candidate vaccine elicited neutralizing B-cell and T-cell activities and protected animals against homologous and heterologous hPIV-3 challenges. These hPIV-3 results confirmed and supplemented our previous studies showing that SeV recombinants expressing either RSV G or RSV F could protect against RSV [22;23;25;38].

We also tested a mixture of two recombinants (the rSeV-RSV F and rSeV-hPIV3-HN constructs) expressing RSV and PIV-3 proteins in cotton rats. The mixed candidate vaccine completely protected cotton rats against challenge with three different pathogens: hPIV-1, hPIV-3 and RSV. The full protection against hPIV-3 and RSV was dependent on the presence of PIV-3 HN and RSV F genes, respectively. However, SeV alone was sufficient to protect completely against hPIV-1 (as previously demonstrated [14]) and partially against hPIV-3.

The precise contributions of B-cell and T-cell activities to protection against the hPIVs and RSV were not dissected in the current study, but will be a topic of future research. It is likely that both B-cells and T-cells contributed to the successful outcome. PIV-specific antibodies are known to provide a first line of defense against virus infection by preventing virus entry into target host cells [31;32;39], while T-cells may provide back-up mechanisms by secreting cytokines and lysing virus-infected cells [40]. Research in a mouse model has shown that antibodies or T-cells activated by an SeV-based RSV F vaccine can each reduce viral load by approximately 2 logs or more after an RSV challenge [41]. Today's most efficacious, licensed vaccines generally elicit a combination of B-cell and T-cell responses [42-46]. Vaccines that elicit only T-cell activity in the absence of antibody have often failed to provide complete protection in pre-clinical and clinical studies [47-49].

Results in the present report encourage further testing of both unmodified and recombinant SeV vaccines [38]. In 1952, when SeV was first isolated in Sendai Japan, it was thought to be the etiologic agent of a human respiratory disease, but this conclusion was discounted by leaders in the field in later years [1]. Five decades have passed since the discovery of SeV, and there remains no evidence of SeV-related infection or disease in humans. SeV therefore holds great clinical appeal, particularly because the vaccine can be administered to the target pediatric population (≤ 12 months of age, [50]) without needle sticks.

Our own clinical trials have thus far shown unmodified SeV to be safe in adults [17]. No serious vaccine-related adverse events have been observed in any of our study participants. Live SeV was not isolated from these volunteers after vaccination, a reflection of pre-existing hPIV-specific immune responses in adults. Clinical trials in a younger volunteer population are ongoing.

The absence of serious vaccine-related adverse events is predicted in humans, because SeV is a mouse pathogen and is host-range restricted. This is in part due to the unique sensitivity of SeV to the innate immune activities elicited by human interferon [16]. As demonstration of viral sensitivity, Bousse et. al. showed that unlike hPIV-1, SeV could not overcome IFN-mediated growth suppression in human lung cells. As further suggestion of SeV safety and

efficacy in primates, we and others showed that the virus caused no disease in either African Green Monkeys (AGM) or chimpanzees, yet conferred complete protection against hPIV-1 (in AGM, protection was superior to that conferred by hPIV-1 itself [14;15]). Also, when the peak growth of SeV in the lower respiratory tract (LRT) of chimpanzees was determined, it was found to be less than that of bPIV-3 (a vaccine that appears to be safe in human infants [4]). Specifically, the peak tracheal lavage fluid titer in chimpanzees was 10^3 after intratracheal inoculation with 10^4 TCID₅₀ of bPIV-3, but was less than 10^3 after intranasal and intratracheal inoculation with 10^5 TCID₅₀ of SeV (a dose of virus 10 times as great [15;51]). Each of the points stated above encourage the further testing of SeV in clinical trials, the completion of which will fully define vaccine safety in humans.

In conclusion, we have demonstrated that two new SeV constructs are capable of conferring complete protection against hPIV-3 in a cotton rat model. We have also shown that a dual vaccine conferred complete protection against hPIV-1, hPIV-3 and RSV. Such a dual vaccine may eventually protect human infants from three serious respiratory pathogens; the xenogenic SeV backbone may protect from hPIV-1 while the passenger genes may protect from both hPIV-3 and RSV. Clearly, a single intranasal inoculation that can target multiple respiratory pathogens would offer great benefit in clinical pediatrics.

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Figure 1. Production and testing of recombinant Sendai viruses

A) The rSeV genome is shown with an engineered NotI site. B) The hPIV-3-F gene, an SeV transcription termination sequence and an SeV transcription initiation sequence were cloned into the NotI site to create rSeV-hPIV3-F. C) The hPIV-3-HN gene, an SeV transcription termination sequence and an SeV transcription initiation sequence were cloned into the NotI site to create rSeV-hPIV3-HN. D, E) Immunoprecipitations were performed to demonstrate F and HN expression by cells infected with rSeV-hPIV3-F and rSeV-hPIV3-HN. Experiments involved the infection of HEP-2 cells with recombinant SeV. Sixteen hours later, cells were labeled with [³⁵S] Promix. F protein (panel A) was immunoprecipitated with a polyclonal antibody specific for the cytoplasmic tail of hPIV-3 F. HN protein (panel B) was

immunoprecipitated with a polyclonal antibody specific for the cytoplasmic tail of hPIV-3 HN. Wild-type (wt) SeV and hPIV-3 were used as negative and positive controls, respectively.

Figure 2. Recombinant SeV immunizations elicit hPIV-3-specific antibodies

Groups of 5 cotton rats were inoculated with 2×10^6 PFU rSeV-hPIV3-F, rSeV-hPIV3-HN, a combination of the two vaccines (each at 1×10^6 PFU), unmodified SeV or PBS. Sera collected 4 weeks later were pooled, serially diluted, and tested by ELISA for hPIV-3-specific antibody activity. Absorbance values are shown with standard error bars (5 replicates per test). Individual sera were also tested and yielded similar results. (A) ELISAs were conducted with serially diluted samples on plates coated with hPIV-3 lysate. (B) ELISAs were conducted with serially diluted samples on plates coated with wild-type SeV lysate.

A. F peptide pool

POOL 1
 MPTSILLIITTIMASFCQIDITKLQHVGLVNSPKGMKISQNFETRYLILSLIPKIEDSNSCGDQIQKQ
POOL 2
 YKRLDLRLIIPLYDGLRLQKDVIVSNQESNENTDPRTKRFFGGVIGTIALGVATSAQITAALVEAKQA
POOL 3
 RSDIEKLKEAIRDTNKAVQSVQSSIGNLIVAIAKSVQDVYVKEIVPSIARLGCEAAGLQGLTQHYSEL
POOL 4
 TNIFGDNIGSLQEKGIKLGASLYRTNITEIFTTSTVDKYDIYDLLFTESIKVRVIDVLDNDYSITLQV
POOL 5
 RLPLLTALLNTQIYKVDISISYNIQNRWEYIPLPSHIMTKGAFLGGADVKECIEAFSSYICPSDPGFVLNH
POOL 6
 EMESCLSGNISQCPRTVVTSDIVPRYAFVNGGVVANCITTTCTCNGIGNRINQPPDQGVKIITHKECNTI
 GINGMLFNTNKEGTALAFYTPNDITLNNVALDPIDISIELNKAASDLEESKEWIRRSNQKLDISGNWHQS
POOL 6
 STTIIIVLIMIIILFIINVTIIIIAVKYYRIQKRNRVDQNDKPYVLTK

B. HN peptide pool

POOL 1
 MEYWKHTNHGKDAGNELETSMAHGNKIKITVILWTLIIVLLSIVFIIVLINSIKSEKAHESLLQDVN
POOL 2
 NEFMEVTEKIQMASDNINDLIQSGVNTLLTIQSHVQNYIPISLTQQMSDLRKFISEITIRNDNOEVPPQ
POOL 3
 RITHDVGIKPLNPDFFWRCTSGLPMLKTPKIRLMPGPGLLAMPPTVDGCVRTPSLVINDLIYAYTSNLI
POOL 4
 TRGCQDIGKSYQLGIIITVNSDLVPDLNPRISHTFNINDNRKSCSLALLNTDVYQLCSTPKVDERSDY
POOL 5
 ASSGIEDIVLDIVNHGDSISTTRFKNNNISFDQPYAALYPSVGPGEIYYKGIIFLGYGGLHHPINENAIC
POOL 6
 NTTGCPGKTQRCNQASHSPWFSDDRMVNSIIIVDKGLNSIPKLKVWTISMRQNYWGSEGRLLLLGNKIY
 IYTRSTSWHSLQLGLIIDITDYSIRIKWTWHNVLSRPGNECPWGHSCPDGCITGVYTDAYPLNPTGSI
POOL 6
 VSSVIDSQKSRVNFVIITYSTATERNVLAIRNKTLSAGYTTTSCITHYNGKGYCFHIVEINHKSLLDTFQPM
 LFKTEIPKSCS

C. rSeV-hPIV3-F primed

E. SeV-primed

D. rSeV-hPIV3-HN primed

F. SeV-primed

Figure 3. T-cells respond to peptide pools representing hPIV-3 F and HN sequences

The synthesized peptides were generally 15 amino acids in length and were initiated at 10-amino acid intervals to cover the length of the hPIV-3 F or HN protein. Each pool included 10 peptides. Overlapping peptide pools are shown in panels A and B by alternating bold and underlined sequences. T-cell tests involved the isolation of mediastinal lymph nodes (MLN) from cotton rats 10 days after animal inoculation with rSeV-hPIV3-F (Panel C), rSeV-hPIV3-HN (panel D), or unmodified SeV (panels E and F) to assess T-cell function with the IFN- γ ELISpot assay. MLN were combined from each group (≥ 3 animals per group) and tested against each of 6 peptide pools or against no peptide. Cells plated per well were 0.5×10^6 for panels C and D, and 1×10^6 for panels E and F. Values are the mean $\pm$ standard error.

Figure 4. Recombinant viruses confer protection against challenges with either homologous or heterologous hPIV-3

Five weeks after immunizations with rSeV-hPIV3-F, rSeV-hPIV3-HN, or a 1:1 mixture of the two vaccines, at a final dose of 2×10^6 PFU/cotton rat, animals were challenged with the homologous virus (C243, 2×10^6 PFU/cotton rat, panel A) or a heterologous virus (8-94, 1.5×10^6 PFU/cotton rat, panel B). Pulmonary virus loads were measured on day 3 post-challenge. Each symbol represents the pulmonary titer (TCID₅₀) in an individual cotton rat. Control animals received wild-type SeV (2×10^6 PFU/cotton rat) or PBS instead of recombinant vaccines.

A. RSV Protection

B. hPIV-3 Protection

C. hPIV-1 Protection

Figure 5. Mixed rSeV-hPIV3-HN and rSeV-RSV-F recombinant SeV vaccines confer protection against challenges with hPIV-1, hPIV-3 and RSV

Five weeks after immunizations with a vaccine mixture (2×10^6 PFU rSeV-RSV-F and 2×10^6 PFU rSeV-hPIV3-HN per cotton rat), groups of 5 animals were challenged with RSV (A2, 1.5×10^6 PFU/cotton rat, top panel), hPIV-3 (C243, 2×10^6 PFU/cotton rat, middle panel) or hPIV-1 (C35, 2×10^6 PFU/cotton rat, bottom panel). Cotton rats immunized with wild-type SeV (2×10^6 PFU/cotton rat) or PBS instead of recombinant vaccines were used as controls. Viral loads were measured in animal lungs on day 3 post-challenge. RSV and hPIV-1 viral loads were measured as PFU/cotton rat, while hPIV-3 viral loads were measured as TCID₅₀/cotton rat. Each symbol represents an individual animal.

Table 1
Recombinant SeV vaccines elicit PIV-3-specific antibodies with cross-reactive neutralizing capacity

Percent neutralization of hPIV-3 isolates:					
Imm.	Serum dilution	C243	4-04	5-97	8-94
F	1:16	100	ND	ND	ND
HN		100	ND	ND	ND
F+HN		100	ND	ND	ND
SeV	1:64	83	ND	ND	ND
PBS		0	ND	ND	ND
F		100	100	100	100
HN		100	100	100	100
F+HN		100	100	100	100
SeV		0	0	0	0
PBS		0	0	0	0
F		83	100	100	100
HN		100	100	100	100
F+HN		100	100	100	100
SeV	1:256	0	0	0	0
PBS		0	0	0	0
F		83	100	100	100
HN		100	100	100	100
F+HN		100	100	100	100
SeV		0	0	0	0
PBS		0	0	0	0
F		0	0	17	17
HN		100	100	100	83
F+HN		100	100	83	50
SeV	1:1024	0	0	0	0
PBS		0	0	0	0

Legend. Pooled serum samples were prepared from cotton rats inoculated with rSeV-hPIV3-F, rSeV-hPIV3-HN, a combination of the two vaccines, unmodified SeV or PBS. Tested viruses were the homologous C243 (ATCC) and the heterologous 4-04, 5-97 and 8-94 (kindly provided by Dr. Randy Hayden). Sera were mixed with virus for one hour and then inoculated onto LLC-MK₂ cell monolayers. Neutralization is scored as the percent reduction of wells with hemagglutination activity. Imm.=Immunogen. ND=Not Determined.