Published in final edited form as:

Vaccine. 2014 May 30; 32(26): 3264–3273. doi:10.1016/j.vaccine.2014.03.088.

Sendai virus-based RSV vaccine protects against RSV challenge in an in vivo maternal antibody model

Bart G. Jones^a, Robert E. Sealy^a, Sherri L. Surman^a, Allen Portner^a, Charles J. Russell^{a,b}, Karen S. Slobod^c, Philip. R. Dormitzer^c, John DeVincenzo^{b,d,e}, and Julia L. Hurwitz^{a,b,f}

^aDepartment of Infectious Diseases, St. Jude Children's Research Hospital, 262 Danny Thomas Place, Memphis, TN USA

^bDepartment of Microbiology, Immunology and Biochemistry, University of Tennessee Health Science Center, Memphis, TN USA

^cNovartis Vaccines and Diagnostics, Cambridge, Massachusetts USA

^dChildren's Foundation Research Institute at Le Bonheur Children's Hospital, Memphis, TN USA

^eDepartment of Pediatrics, University of Tennessee Health Science Center, Memphis, TN USA

Abstract

Respiratory syncytial virus (RSV) is the cause of significant morbidity and mortality among infants, and despite decades of research there remains no licensed vaccine. SeVRSV is a Sendai virus (SeV)-based live intranasal vaccine that expresses the full length RSV fusion (F) gene. SeV is the murine counterpart of human parainfluenza virus type 1. Given that the target population of SeVRSV is young infants, we questioned whether maternal antibodies typical of this age group would inhibit SeVRSV vaccine efficacy. After measuring SeV- and RSV-specific serum neutralizing antibody titers in human infants, we matched these defined titers in cotton rats by the passive transfer of polyclonal or monoclonal antibody products. Animals were then vaccinated with SeVRSV followed by a three month rest period to allow passively transferred antibodies to wane. Animals were finally challenged with RSV to measure the *de novo* vaccine-induced immune responses. Despite the presence of passively-transferred serum neutralizing antibodies at the time of vaccination, SeVRSV induced immune responses that were protective against RSV challenge. The data encourage advancement of SeVRSV as a candidate vaccine for the protection of children from morbidity and mortality caused by RSV.

Keywords

Respiratory syncytial virus vaccine; Sendai virus; maternal antibody model; neutralizing antibodies; protective immunity

© 2014 Elsevier Ltd. All rights reserved.

^fCorresponding Author: Julia L. Hurwitz, Department of Infectious Diseases, St. Jude Children's Research Hospital, 262 Danny Thomas Place, Memphis, TN 38105, Phone: 901-595-2464, FAX: 901-595-3099, address: julia.hurwitz@stjude.org.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

INTRODUCTION

RSV constitutes a serious threat to the world's children, causing more than 150,000 deaths each year [1]. The most serious disease occurs upon a child's first exposure to RSV, suggesting that natural infection confers a degree of protective immunity [2–4]. Most vulnerable to RSV disease are young infants, premature children, children with congenital heart or lung disease, and the immunocompromised [5]. Despite decades of research, the only proven method of prophylaxis is administration of preformed antibodies. Because such prophylaxis is expensive and logistically difficult to administer, it is often unavailable to the high-risk infants who need it most [6–8]. In the USA, less than 3% of the birth cohort is eligible to receive passive antibody prophylaxis [9]. A safe and effective RSV vaccine has not yet been developed [10–12].

We are currently advancing SeVRSV, an intranasal (i.n.) recombinant Sendai virus (SeV)-based vaccine produced by reverse genetics to express the gene for the RSV fusion protein (F). SeV is a parainfluenza virus type 1 (PIV-1) of mice, and can therefore serve as both an RSV F gene delivery vehicle and a Jennerian vaccine for human parainfluenza virus type 1 (hPIV-1; the major cause of croup in children) [13–15]. Our pre-clinical research has demonstrated that SeV and SeVRSV, when delivered by the i.n. route, safely protect non-human primates from hPIV-1 and RSV, respectively [14;16]. Furthermore, SeVRSV protects small animals against both RSV A and B isolates, a reflection of the high conservation of the RSV F gene [15]. SeV-based vaccines confer protection following a single i.n. inoculation, and protect for the animal's lifetime [17]. The i.n. SeV vaccine backbone has already advanced to clinical studies and has been well tolerated in adults [18] and in 3–6 year old children.

Because RSV causes serious disease in young infants, it would be advisable to immunize children by 2 months of age, at the time of other standard immunizations with polio virus, tetanus, diphtheria and rotavirus vaccines. A concern therefore arises as to whether RSV or PIV-1-specific maternal antibodies, present in human infants at 2 months of age, may inhibit vaccine efficacy. Maternal antibodies may influence vaccine amplification and respective immune responses by a number of distinct mechanisms including classical virus neutralization, virus aggregation, antibody-dependent cell-mediated cytotoxicity, and modulation of innate or adaptive immune effector functions [19–25]. To address the concern that maternal antibodies might inhibit SeVRSV vaccine efficacy, we conducted experiments to (i) measure levels of RSV and SeV-specific serum neutralizing antibodies in human infants, (ii) recapitulate this defined clinically relevant range of neutralizing titers within cotton rats by passive antibody transfers, and (iii) measure the protective capacity of SeVRSV in cotton rats in the presence of antibodies. Results showed that SeVRSV vaccine efficacy was not perturbed in the presence of antibodies representative of the 2 month old human infant. Despite the presence of passively-transferred neutralizing antibodies at the time of vaccination, SeVRSV-induced *de novo* virus-specific neutralizing antibodies and conferred protection against RSV challenge three months after vaccination. Data encourage the advancement of SeVRSV to clinical testing for the potential protection of infants from RSV disease.

MATERIALS AND METHODS

Infant samples

Sera were collected from hospitalized infants at Le Bonheur Children's Hospital, Memphis, TN for testing of neutralizing antibodies. Collections were with approval from the appropriate Institutional Review Boards with jurisdiction over Le Bonheur Children's Hospital and St. Jude Children's Research Hospital. Samples were from three groups of previously healthy, term, hospitalized infants without known immune system abnormalities: a) Infants hospitalized due to an apparent respiratory infection, with a positive test for RSV by real-time RT-PCR, b) Infants hospitalized due to an apparent respiratory infection with a negative test for RSV by real-time RT-PCR, and c) Infants hospitalized for reasons other than respiratory disease and unrelated to the respiratory tract. Samples were from age groups '0 to 2 months' (4–61 days, n= 33), '2 to 4 months' (62–122 days; n=31), and '4 to 6 months' (123–183 days, n=23).

Antibodies for passive transfer

The polyclonal antibodies used in passive transfer experiments to model maternal antibodies included RespiGam and GAMUNEX. RespiGam, although no longer marketed, was developed by MedImmune (Gaithersburg, MD) as a polyclonal preparation of adult human antibodies for prophylaxis against RSV. This product was produced by first screening blood donors with an RSV microneutralization assay. Donor plasma with the highest anti-RSV antibody neutralization titers (induced by natural RSV exposures) were selected and pooled into lots from which intravenous immune globulin was manufactured. Monthly RespiGam infusions have been shown to significantly reduce RSV-related hospitalizations in high risk infants [26–29]. GAMUNEX is a second polyclonal human immune globulin product used to treat primary humoral immunodeficiencies (Talecris Biotherapeutics, Inc. Research Triangle Park, NC).

Monoclonal antibody preparations included anti-SeV-specific monoclonals (a 1:1 mixture by weight of two neutralizing mouse monoclonal antibodies (S2, M57) generated against the SeV hemagglutinin-neuraminidase (HN) protein [30]), and Palivizumab, a humanized monoclonal antibody with specificity for the RSV F protein, used to prevent RSV disease in high-risk patients (MedImmune, Gaithersburg, MD). Antibodies were administered to cotton rats 2 days prior to vaccination, as described below.

Vaccination and challenge experiments

The basic strategy for vaccine testing in the maternal antibody model is shown in Figure 1. Experiments were designed to model planned, future clinical trials in which a dose of $1-2 \times 10^7$ SeVRSV will be tested in 2 month old infants. Female adult cotton rats (*Sigmodon Hispidus*, Harlan) received antibodies by passive transfer 2 days prior to vaccination. Generally, antibodies were administered by the IP route in a 2.0 ml volume. Animals were bled immediately prior to vaccination for measurement of passively-transferred serum neutralizing antibody levels. Test animals were immunized intranasally (i.n.) with SeVRSV in 100 μ l PBS ($1-2 \times 10^7$ EID₅₀). Approximately three months later, cotton rats were bled for analyses of vaccine-induced neutralizing antibody levels and challenged i.n. with $1.5 \times$

10⁶ plaque forming units (pfu) RSV-A2 in 100 µl volume. After 3–4 days, cotton rats were sacrificed and lungs were removed for virus titrations. The experimental design was based on literature demonstrating that peak virus load occurs 2–4 days after virus challenge in animals that were previously vaccinated [31–33]. Experiments were conducted in replicate to ensure reproducibility. In rare cases, animals that received passive antibody transfers did not exhibit detectable neutralizing activities in sera 2 days later. These animals were omitted from analyses, as the possibility of suboptimal passive antibody administration to these animals could not be excluded.

Microneutralization assays

Human sera were inactivated with heat (56°C for 45 min) prior to testing in RSV microneutralization assays. Human sera were inactivated with receptor destroying enzyme (RDE (II) Denka Seiken Co., LTD Tokyo, Japan, distributed by Accurate Chemical and Scientific Corp, Westbury, NY) followed by heat prior to testing in SeV microneutralization assays.

RSV microneutralization assays—Assays were performed with human or cotton rat sera based on the method of Anderson et. al. [34;35]. Sera were tested at a starting dilution of 1:40 with two-fold serial dilutions from 1:40 to 1:2560. There were 4–6 replicates per test or control sample in 96-well flat-bottomed plates (100 µl/well). To each well was added 50 µl RSV-A2 (50–100 pfu per well) with mixing. Controls included wells with no virus and wells with virus, but no antibody. Medium was DMEM, 10% FBS, glutamine, and gentamicin (50 µg/ml). After plates were incubated for 1 hour at 37°C, 5% CO₂, washed Hep2 cells were added to plates at 2×10^4 cells/well. After a further 4 day incubation at 37°C, 5% CO₂, supernatants were aspirated and wells were washed for development by ELISA. To conduct ELISAs, washed cells were fixed for 1 min with 100 µl 80:20 acetone (Fisher)/DPBS (DPBS with MgCa, Lonza). Wells were washed again and blocked with 100 µl 1% BSA (Sigma/Aldrich) in DPBS for 1 hour at room temperature. Next, wells were incubated with an anti-RSV antibody conjugated to horse radish peroxidase in 1% BSA/DPBS. After a 1 hr incubation at room temperature, plates were washed and developed with 100 µl TMB peroxidase (KPL)/well. Following color development, reactions were stopped with 100 µl 4N H₃PO₄ and plates were read on a microplate reader at O.D. 450 nm.

SeV microneutralization assays—Sera were diluted serially as above, starting at 1:40 with two-fold dilutions to 1:2560 (100 µl/well). To each well was added 50 µl SeV (approximately 10 TCID₅₀ per well). After a 1 hour incubation, samples and controls were transferred to 96-well plates, to which LLC MK₂ cells had been added the previous day and cultured overnight (2×10^5 cells/ml 100 µl/well). After an additional overnight incubation, supernatants were removed from wells and 200 µl media were added (DMEM with glutamine, gentamicin, 0.1% BSA and acetylated trypsin (2–4 µg/ml)). Plates were then incubated for an additional 3 days at 37°C, 5% CO₂. Plates were developed by ELISA as above, but with SeV-specific antibodies rather than RSV-specific antibodies, conjugated to horse radish peroxidase in DPBS with 1% BSA.

Titers were defined as the highest serum dilution that resulted in at least ½ wells exhibiting an O.D. reduction of 50% compared to positive controls. A score of >2560 was given when the majority of test wells exhibited an O.D. reduction of 50% at the 1:2560 sample dilution. It should be noted that microneutralization assays for RSV-specific and SeV-specific antibodies differed by several variables including virus input, mammalian host cell, and developing antibody reagents. Therefore, absolute neutralization titers could be compared within assays, but not between assays.

ELISA for the detection of passively transferred human antibodies in cotton rats

ELISA plates were incubated with disrupted RSV-A2. After plates were washed and blocked with PBS/1% BSA, serum samples (diluted 1:1000) were incubated on plates for at least three hours or overnight. Plates were washed and incubated with HRP-conjugated goat anti-human IgG (non-cross-reactive with cotton rat antibodies) for at least 1 hr. Assays were developed with TMB substrate for O.D. reading at 450 nm.

Virus titrations

Lungs were homogenized in 5.0 ml DPBS for the measurement of SeVRSV titers. Ten-fold serial dilutions were performed in DMEM supplemented with gentamicin, glutamine, and 5 ug/ml acetylated trypsin for testing. Diluted samples were incubated on LLC-MK₂ cell monolayers in 96-well plates with 8 replicates per dilution at 33°C for 5 days. 50 ul supernatant samples from each well were then removed and mixed with 50 ul chicken RBC for incubation at 4°C for 45 min. Plates were scored for hemagglutination and TCID₅₀ values were calculated using the Reed-Muench formula.

For titrations of RSV, samples were serially diluted in EMEM/0.1% BSA, and plated on Hep2 cells in 6-well plates. Plates were incubated at 37°C, 5% CO₂ overnight after which the viral inocula were removed and replaced with 1.0 ml/well 0.75% methylcellulose/EMEM/10% FCS. Plates were incubated at 37°C, 5% CO₂ for an additional 4–5 days and aspirated. Wells were washed with DPBS and fixed with formalin. Cells were stained with Hematoxylin and Eosin Y and washed, after which plaques were counted.

RESULTS

Maternal antibody neutralization titers against SeV and RSV in infants

A study was designed and approved by institutional review boards to collect sera from inpatient infants at Le Bonheur Children's Hospital (Memphis, TN) for testing in RSV-specific and SeV-specific neutralization antibody assays. SeV-specific antibodies are present among maternal antibodies, because most humans are exposed repeatedly to hPIV-1, a virus closely related to SeV [36]. In Table 1 are shown neutralization titers from 14 representative serum samples in age groups 0 to 2 months, 2 to 4 months, and 4 to 6 months. Titers against both SeV and RSV were highest within the youngest age group, as expected due to the decay of maternal antibodies after birth. A nadir of neutralizing antibodies is generally reached at 2 months of age. Levels then rise as infants are naturally exposed to RSV and generate an endogenous immune response [4;37] Lewis, 2005 4032/id}. When antibodies in infants fall below a titer of 100 in the RSV neutralization assay, infants are particularly

vulnerable to RSV infections and may benefit most from RSV prophylaxis [37]. This concept is re-emphasized by results in Table 2 showing that the infants who were hospitalized for RSV infections (confirmed by PCR) rarely exhibited RSV neutralization titers above 100 (3/22 samples), whereas infants admitted to the hospital for reasons other than RSV infections often exhibited RSV neutralization titers above 100 (25/57, $p < .02$).

A maternal antibody model in cotton rats for SeVRSV testing

Based on the results described above, we designed a cotton rat maternal antibody model to determine if SeVRSV would retain its protective capacity in the presence of serum antibody titers typical of the '2 to 4 month old' age group, the target population for SeVRSV vaccination. The model, illustrated in Figure 1, involved the passive transfer of antibodies into cotton rats to recapitulate serum antibody titers of the target human infant. Two days later, animals were vaccinated with SeVRSV and three months later, animals were tested for vaccine-induced protection against an RSV challenge.

After a three month rest period, passively transferred antibodies fail to inhibit RSV infection

As a first test of the maternal antibody model, we proposed to passively transfer RespiGam into cotton rats and then vaccinate cotton rats with SeVRSV 2 days later to determine if SeVRSV could induce a *de novo*, protective immune response against RSV. Since RespiGam could also contribute directly to RSV neutralization, the test of vaccine-induced protection could not be performed until RespiGam had waned. To define the time interval needed for RespiGam to dissipate, we sampled cotton rat sera longitudinally after RespiGam injections. An ELISA was used that specifically measured human antibodies and did not cross-react with cotton rat antibodies. As shown in Figure 2A, animals injected with RespiGam exhibited a progressive loss of antibody over 21 days. After 3 months, human antibodies were below detection in all animals (Figure 2B). Based on these data, we rested animals for 3 months between passive antibody transfers and RSV challenges in all vaccine experiments.

To confirm that RespiGam could not protect against RSV challenge after a 3 month rest interval, test animals were injected with RespiGam, but were not vaccinated. Upon RSV challenge 3 months later, the animals that received RespiGam without SeVRSV vaccination were not protected from challenge (Figure 3). In addition, when unvaccinated animals were injected with GAMUNEX (100 mg), a second preparation of human polyclonal antibodies (described below and in the Materials and Methods section) and rested for 3 months, animals were not protected from RSV challenge. Confirming the waning of administered pre-formed antibodies, all animals injected with either RespiGam or GAMUNEX and challenged with RSV 3 months later, exhibited virus titers exceeding 1×10^4 pfu/animal 3–4 days post-challenge.

SeVRSV is efficacious in the presence of RespiGam

The cotton rat model was used to test SeVRSV vaccine efficacy in the presence of RespiGam. For these experiments, test cotton rats were grouped to receive RespiGam at one of two doses (1 mg or 10 mg). Two days later, test animals were vaccinated with SeVRSV.

A dose of $1-2 \times 10^7$ EID₅₀ SeVRSV was used to match the dose intended for future testing in humans. Control animals received no antibody and/or no vaccine. On the day of vaccination, blood was collected to test serum neutralizing antibody levels against SeV or RSV. Based on data described above, cotton rats were rested for 3 months to ensure decay of passively transferred antibodies and respective protection against RSV challenge. At the 3 month time point, blood was collected to assess *de novo* vaccine-induced SeV- or RSV-specific neutralizing antibodies, after which animals received an i.n. RSV challenge. Animals were sacrificed 3–4 days later for measurement of RSV (virus) titers in the lung.

Results are shown in Figures 4A and 4B. As shown in Figure 4A, 2 days after RespiGam administration, RSV-specific serum neutralizing titers ranged from 40–80 in animals receiving the 1 mg RespiGam dose and from 640–1280 in animals receiving the 10 mg RespiGam dose. Neutralizing SeV-specific antibodies were undetected at the lowest 1:40 serum dilution of cotton rat sera. These values reflected the design of RespiGam in that antibody preparations had been pre-selected for high RSV neutralization capacity. For animals that received the 10 mg dose, RSV neutralizing titers exceeded those typical of the 2 month old infant target population (Table 1).

As shown in Figure 4B, all vaccinated animals were protected from subsequent challenge with RSV, regardless of whether they were vaccinated in the presence or absence of RespiGam. Administration of the two RespiGam doses (1 and 10 mg) allowed vaccine testing in animals with a range of RSV neutralizing titers that mimicked the range of titers among target infants (measured in the accompanying sero-epidemiologic analysis, Table 1). Animals that were vaccinated after receiving the 0 or 1 mg dose of RespiGam (open circles and closed diamonds respectively in Figure 4B) were fully protected from RSV, while animals that were vaccinated after the 10 mg dose of RespiGam (triangles) exhibited an average two log reduction of challenge virus compared to unvaccinated controls (solid circles). Animals that received RespiGam without vaccine were not protected (Figure 3). In previous studies of prophylactic agents in cotton rats, a two log reduction of RSV titers in test animals translated to a significant reduction of pediatric hospitalizations [38;39].

The degree of protection was not fully correlated with the vaccine-induced RSV-specific antibody neutralizing titers present immediately prior to challenge (Figure 4A). For example, animal #13, a recipient of 10 mg RespiGam, exhibited an anti-RSV antibody titer of 160 and experienced incomplete protection from challenge, whereas recipients of 1 mg RespiGam (animals #9, 10 and 11) exhibited the same RSV-titer (160), but achieved complete protection from RSV. Results suggested that vaccine-induced B and T cell effector functions other than classical neutralization contributed to protection [15;15;19;22–24;40–44]. In total, results from RespiGam experiments demonstrated that SeVRSV is an effective vaccine in the cotton rat maternal antibody model when RSV-specific serum neutralizing antibodies are at levels matching those observed in the 2 month old human target population.

SeVRSV is efficacious in the presence of GAMUNEX

To determine if the results with RespiGam could be generalized to other human polyclonal antibody preparations, we repeated tests with GAMUNEX used as a substitute for RespiGam. GAMUNEX is a clinical grade immune globulin product used to supplement

immunodeficient human patients. It is manufactured similarly to RespiGam, but blood donors are not pre-selected for high titers of RSV-specific neutralizing antibodies. Experiments were again designed to (i) passively transfer antibodies into cotton rats, in this case at doses of 10 mg, 25 mg and 100mg, (ii) measure serum neutralizing activities and vaccinate with SeVRSV 2 days later, (iii) measure *de novo* vaccine-induced RSV-specific neutralizing antibodies in the presence of antibodies and challenge three months later, and (iv) measure RSV virus loads in cotton rat lungs at 3–4 days post-challenge to evaluate vaccine efficacy.

Figure 5A illustrates RSV- and SeV-specific neutralizing antibodies present in cotton rats 2 days after passive transfer. As shown in Figure 5B, vaccinated animals were fully protected from RSV challenge in groups that received the 10 mg or 25 mg dose of GAMUNEX. Animals that were vaccinated after receiving the 100 mg dose of GAMUNEX were also significantly, but in this case incompletely protected from RSV. As in RespiGam experiments, the SeVRSV-induced serum RSV-specific neutralizing activity measured immediately prior to RSV challenge (Figure 5A) was not entirely predictive of the degree of RSV protection. In fact, in some cases there was protection when neutralizing activity was below detection. Results again support the suggestion that endogenous vaccine-induced effector functions other than measureable serum antibody neutralization contributed to the protective effect [15;42;43].

SeV-based vaccines typically grow transiently in the respiratory tract, a feature that lends to their immunogenicity [14;16]. Given that SeVRSV retained its protective capacity in the presence of passively transferred antibodies, we predicted that antibodies would not interfere with transient vaccine amplification. To test this concept, we measured SeVRSV virus titers in the respiratory tract 4 days after i.n. vaccine inoculation. Figure 6 compared SeVRSV titers among test animals that received GAMUNEX prior to vaccination and control animals that received no GAMUNEX. As predicted based on vaccine efficacy, passively transferred antibodies did not interfere with measureable SeVRSV amplification.

Vaccine efficacy in the presence of monoclonal anti-SeV or anti-RSV specific monoclonal antibodies

The results with the 100 mg dose of GAMUNEX showed that a combination of high-titered anti-SeV (cross reacting with hPIV-1) and anti-RSV antibodies could modestly inhibit SeVRSV vaccine efficacy. A final set of experiments was designed to separate SeV-specific from RSV-specific antibodies to determine if there were differences in SeVRSV vaccine inhibition. To address this, we first tested a combination of two neutralizing, monoclonal antibodies with specificities for the SeV hemagglutinin-neuraminidase (HN) protein. Two doses (0.12 and 0.36 mg) were tested using the same cotton rat protocol as described above to mimic or exceed the neutralizing activities typical of the 2 month old infant. As expected, in pre-vaccine neutralization tests, SeV-specific, but not RSV-specific neutralizing activities could be measured in cotton rats, with greatest activity in animals that received the 0.36 mg antibody dose (Figure 7A). When vaccinated animals were challenged with RSV, all animals administered the 0.12 mg antibody dose were fully protected and three of four animals administered the 0.36 mg dose were fully protected (Figure 7B). The conclusion

from these experiments was that monoclonal anti-SeV antibodies may have had a mild effect on SeVRSV vaccine efficacy, but only at titers well above those observed in the 2 month old infant.

To test isolated RSV-specific antibodies in the maternal antibody model, we used Palivizumab (Synagis), an RSV-F specific neutralizing antibody preparation used routinely for prophylaxis of RSV in high-risk infants. Experimental design was unchanged and doses of 1 mg and 10 mg were tested to determine if neutralizing antibody titers matching or exceeding those of the 2 month old infant would inhibit SeVRSV vaccine efficacy. As shown in Figure 8A, RSV neutralization titers in cotton rats sampled before vaccination were 160 to 320 for animals that received the 1 mg dose (mimicking titers observed in target infants) and >2560 for most animals that received the 10 mg dose. As shown in Figure 8B, all SeVRSV-vaccinated and RSV-challenged animals were protected from RSV. All vaccinated animals generated endogenous RSV-specific antibodies in response to vaccination (Figure 8A), although values trended downward among animals that received the higher doses of Palivizumab.

DISCUSSION

The experiments described in this report tested an RSV vaccine candidate, SeVRSV, in a cotton rat maternal antibody model. Experiments were designed to model future clinical trials in which a dose of $1-2 \times 10^7$ SeVRSV may be tested in 2 month old infants. Results showed that when polyclonal or monoclonal antibodies were injected into cotton rats (achieving titers representative of those measured in 0–2 month old infants) prior to vaccination, the vaccine was nonetheless protective against RSV challenge. SeVRSV vaccine efficacy was demonstrated in all vaccinated animal groups. Even for animals that received the highest doses of polyclonal antibodies prior to vaccination, SeVRSV was protective against RSV challenge. When monoclonal antibodies were used, the anti-RSV antibodies alone (Palivizumab) had no inhibitory effect on vaccine efficacy, even at very high titers (>2560). Similarly, administration of SeV-specific monoclonal antibodies prior to vaccination had little impact on vaccine-induced protection against RSV.

Of note, the SeVRSV vaccine particle lacks the RSV F glycoprotein. Instead, SeVRSV encodes the RSV F gene for expression in infected cells [15]. RSV-specific antibodies are therefore not expected to inhibit the initial infection by SeVRSV, but might facilitate lysis of vaccine-infected cells expressing membrane-bound RSV F, by antibody-dependent cell-mediated cytotoxicity (ADCC)[40;45]. It is likely that the monoclonal antibodies had a lesser effect than polyclonal preparations, because polyclonal antibodies target numerous antigenic sites. Due to their heterogeneity, the polyclonal antibodies serve as a better match for placentally-transferred maternal antibodies. The fact that SeVRSV conferred protection against RSV in the presence of polyclonal antibodies suggests that maternal antibodies will not inhibit the SeVRSV vaccine among infants in the 2 month old age group.

We expect that SeV-based vaccines will provide greatest benefit to individuals with low pre-existing immunity, the individuals who are most vulnerable to respiratory virus infections. The planned clinical study will schedule vaccinations with SeVRSV at 2, 4, and 6 months of

age, consistent with other pediatric vaccine schedules. If there are instances in which maternal antibody titers are unusually high in a subset of 2 month old infants, these infants may benefit more from their booster immunizations than from their primary vaccinations.

Results in this report add to information pertinent to other pediatric vaccines. Numerous licensed vaccine products, both non-replicating and replication-competent (e.g. hepatitis, diphtheria, pertussis tetanus, polio virus, rotavirus) are efficacious when administered to children in early life (albeit the magnitude of the antibody response toward the measles virus vaccine is affected by maternal antibodies [46;47]). The success of the oral poliovirus vaccine is of particular interest, because an oral vaccine must overcome maternal antibodies delivered both trans-placentally and via mother's milk. The i.n. SeVRSV vaccine may have an advantage over the oral polio virus vaccine in humans, because antibodies acquired via mother's milk have better access to the gastrointestinal tract than to respiratory tract tissues. This is because humans, unlike rodents, lack a jejunal and duodenal membrane Fc receptor with which IgG is transcytosed through enterocytes to the sub mucosa and then to the blood. [48]. Systemic antibodies, although capable of bathing many tissues including lower respiratory tract (LRT) tissues, traffic relatively poorly to the upper respiratory tract (URT), the site of SeVRSV immunization. The better protection of LRT compared to URT tissues by systemic antibodies has been demonstrated repeatedly in both cotton rats and humans [49–51].

There are a number of different models that can be used to mimic maternal antibodies, each model with its associated advantages and disadvantages [52;53]. The passive transfer model described here is preferred by many researchers, because adult human serum antibodies can be tested. It is otherwise difficult to recapitulate in an animal model the multiple hPIV-1 and RSV exposures that are experienced by a human throughout childhood and adulthood prior to pregnancy, and that mold the antibody response. An alternative model immunizes pregnant mice or cotton rats. Weaning pups are then assessed for protection against viral challenge [53]. These models pose some difficulties, because the immune systems of rodent pups and human infants do not develop at the same pace, nor are there complete parallels between the developmental permissivity of the different species' placentas at similar developmental time points. There are also differences between humans and rodents as described above in that antibodies from a mother's milk primarily protect the gastrointestinal tract tissues in humans, whereas the same antibodies can be widely dispersed in the nursing pups, potentially extending the time during which maternal antibodies may inhibit vaccine efficacy. It is because of these caveats and because of the vast experience with translational passive transfer tests of polyclonal and Palivizumab monoclonal antibodies in cotton rats [49;54;55] that we selected the passive transfer strategy as a maternal antibody model for tests with SeVRSV.

The results described above complement and extend our previous studies with SeVRSV. Additional attractive features of SeVRSV are that: (i) the SeV backbone is extremely sensitive to human interferon (IFN)-associated responses, explaining, at least in part, its safety profile in humans [56], (ii) SeV-based vaccines induce both humoral and cellular responses, including resident antibody forming cell and T cell responses in the URT [41;57], (iii) SeV-based vaccines support the secretion of antibodies into URT airways [57], (iv)

SeV-based vaccines protect within 7 days after vaccination [57], and (v) SeV-based vaccines induce durable protection without requirement for a booster [17]. Rapid and durable immune responses also typify other replication-competent vaccines, such as the smallpox vaccine that is well known for its induction of protective immunity that can persist for more than 50 years in humans [58]. In total, results encourage advancement of SeVRSV to clinical studies. A success in this area may ultimately curtail the serious morbidity and mortality caused by hPIV-1 and RSV in the pediatric arena.

Acknowledgments

We thank Lisa Harrison for assistance with human samples and data. This study was supported in part by NIH NIAID R01 AI088729 and R01 AI083370, NIH NCI P30 CA21765 and the American Lebanese Syrian Associated Charities (ALSAC).

Reference List

1. Nair H, Nokes DJ, Gessner BD, Dherani M, Madhi SA, Singleton RJ, O'Brien KL, Roca A, Wright PF, Bruce N, Chandran A, Theodoratou E, Sutanto A, Sedyaningsih ER, Ngama M, Munywoki PK, Kartasasmita C, Simoes EA, Rudan I, Weber MW, Campbell H. Global burden of acute lower respiratory infections due to respiratory syncytial virus in young children: a systematic review and meta-analysis. *Lancet*. 2010; 375:1545–55. [PubMed: 20399493]
2. Glezen WP, Taber LH, Frank AL, Kasel JA. Risk of primary infection and reinfection with respiratory syncytial virus. *Am J Dis Child*. 1986; 140:543–6. [PubMed: 3706232]
3. Shinoff JJ, O'Brien KL, Thumar B, Shaw JB, Reid R, Hua W, Santosham M, Karron RA. Young infants can develop protective levels of neutralizing antibody after infection with respiratory syncytial virus. *J Infect Dis*. 2008; 198:1007–15. [PubMed: 18702606]
4. Piedra PA, Jewell AM, Cron SG, Atmar RL, Glezen WP. Correlates of immunity to respiratory syncytial virus (RSV) associated-hospitalization: establishment of minimum protective threshold levels of serum neutralizing antibodies. *Vaccine*. 2003; 21:3479–82. [PubMed: 12850364]
5. Buckingham SC, Quasney MW, Bush AJ, DeVincenzo JP. Respiratory syncytial virus infections in the pediatric intensive care unit: clinical characteristics and risk factors for adverse outcomes. *Pediatr Crit Care Med*. 2001; 2:318–23. [PubMed: 12793934]
6. Simoes EA, Groothuis JR, Carbonell-Estrany X, Rieger CH, Mitchell I, Fredrick LM, Kimpen JL. Palivizumab prophylaxis, respiratory syncytial virus, and subsequent recurrent wheezing. *J Pediatr*. 2007; 151:34–42. [PubMed: 17586188]
7. DeVincenzo J. Passive antibody prophylaxis for RSV. *Pediatr Infect Dis J*. 2008; 27:69–70. [PubMed: 18162943]
8. Boivin G, Caouette G, Frenette L, Carbonneau J, Ouakki M, De SG. Human respiratory syncytial virus and other viral infections in infants receiving palivizumab. *J Clin Virol*. 2008; 42:52–7. [PubMed: 18164233]
9. Report of the Committee on Infectious Diseases. American Academy of Pediatrics; 2009. Red Book.
10. Collins PL, Graham BS. Viral and host factors in human respiratory syncytial virus pathogenesis. *J Virol*. 2008; 82:2040–55. [PubMed: 17928346]
11. Hurwitz JL. Respiratory syncytial virus vaccine development. *Expert Rev Vaccines*. 2011; 10:1415–33. [PubMed: 21988307]
12. Shaw CA, Ciarlet M, Cooper BW, Dionigi L, Keith P, O'Brien KB, Rafie-Kolpin M, Dormitzer PR. The path to an RSV vaccine. *Curr Opin Virol*. 2013; 3:332–42. [PubMed: 23727193]
13. Lyn D, Gill DS, Scroggs RA, Portner A. The nucleoproteins of human parainfluenza virus type 1 and Sendai virus share amino acid sequences and antigenic and structural determinants. *J Gen Vir*. 1991; 72:983–7.
14. Hurwitz JL, Soike KF, Sangster MY, Portner A, Sealy RE, Dawson DH, Coleclough C. Intranasal Sendai virus vaccine protects African green monkeys from infection with human parainfluenza virus-type one. *Vaccine*. 1997; 15:533–40. [PubMed: 9160521]

15. Zhan X, Hurwitz JL, Krishnamurthy S, Takimoto T, Boyd K, Scroggs RA, Surman S, Portner A, Slobod KS. 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. *Vaccine*. 2007; 25:8782–93. [PubMed: 18037543]
16. Jones BG, Sealy RE, Rudraraju R, Traina-Dorge VL, Finneyfrock B, Cook A, Takimoto T, Portner A, Hurwitz JL. Sendai virus-based RSV vaccine protects African green monkeys from RSV infection. *Vaccine*. 2012; 30:959–68. [PubMed: 22119594]
17. Jones B, Zhan X, Mishin V, Slobod KS, Surman S, Russell CJ, Portner A, Hurwitz JL. Human PIV-2 recombinant Sendai virus (rSeV) elicits durable immunity and combines with two additional rSeVs to protect against hPIV-1, hPIV-2, hPIV-3, and RSV. *Vaccine*. 2009; 27:1848–57. [PubMed: 19200447]
18. Slobod KS, Shenep JL, Lujan-Zilbermann J, Allison K, Brown B, Scroggs RA, Portner A, Coleclough C, Hurwitz JL. Safety and immunogenicity of intranasal murine parainfluenza virus type 1 (Sendai virus) in healthy human adults. *Vaccine*. 2004; 22:3182–6. [PubMed: 15297072]
19. Guillems M, Bruhns P, Saeys Y, Hammad H, Lambrecht BN. The function of Fcγ receptors in dendritic cells and macrophages. *Nat Rev Immunol*. 2014; 14:94–108. [PubMed: 24445665]
20. Glezen WP, Paredes A, Allison JE, Taber LH, Frank AL. Risk of respiratory syncytial virus infection for infants from low-income families in relationship to age, sex, ethnic group, and maternal antibody level. *J Pediatr*. 1981; 98:708–15. [PubMed: 7229749]
21. Glezen WP. Effect of maternal antibodies on the infant immune response. *Vaccine*. 2003; 21:3389–92. [PubMed: 12850346]
22. Corthesy B, Kraehenbuhl JP. Antibody-mediated protection of mucosal surfaces. *Curr Top Microbiol Immunol*. 1999; 236:93–111. [PubMed: 9893357]
23. Ravetch JV, Clynes RA. Divergent roles for Fc receptors and complement in vivo. *Annu Rev Immunol*. 1998; 16:421–32. [PubMed: 9597136]
24. Sulica A, Morel P, Metes D, Herberman RB. Ig-binding receptors on human NK cells as effector and regulatory surface molecules. *Int Rev Immunol*. 2001; 20:371–414. [PubMed: 11878510]
25. Stensballe LG, Ravn H, Kristensen K, Meakins T, Aaby P, Simoes EA. Seasonal variation of maternally derived respiratory syncytial virus antibodies and association with infant hospitalizations for respiratory syncytial virus. *J Pediatr*. 2009; 154:296–8. [PubMed: 19150677]
26. Groothuis JR, Simoes EA, Levin MJ, Hall CB, Long CE, Rodriguez WJ, Arrobio J, Meissner HC, Fulton DR, Welliver RC. The Respiratory Syncytial Virus Immune Globulin Study Group. Prophylactic administration of respiratory syncytial virus immune globulin to high-risk infants and young children. *N Engl J Med*. 1993; 329:1524–30. [PubMed: 8413475]
27. Groothuis JR, Hoopes JM, Hemming VG. Prevention of serious respiratory syncytial virus-related illness. II: Immunoprophylaxis. *Adv Ther*. 2011; 28:110–25. [PubMed: 21318605]
28. Rodriguez WJ, Gruber WC, Groothuis JR, Simoes EA, Rosas AJ, Lepow M, Kramer A, Hemming V. Respiratory syncytial virus immune globulin treatment of RSV lower respiratory tract infection in previously healthy children. *Pediatrics*. 1997; 100:937–42. [PubMed: 9374560]
29. Groothuis JR, Simoes EA, Hemming VG. Respiratory Syncytial Virus Immune Globulin Study Group. Respiratory syncytial virus (RSV) infection in preterm infants and the protective effects of RSV immune globulin (RSVIG). *Pediatrics*. 1995; 95:463–7. [PubMed: 7700741]
30. Portner A. The HN glycoprotein of Sendai virus: analysis of site(s) involved in hemagglutinating and neuraminidase activities. *Virology*. 1981; 115:375–84. [PubMed: 6274088]
31. Prince GA, Prieels J-P, Slaoui M, Porter DD. Pulmonary lesions in primary respiratory syncytial virus infection, reinfection, and vaccine-enhanced disease in the cotton rat (*Sigmodon hispidus*). *Lab Invest*. 1999; 79:1385–92. [PubMed: 10576209]
32. Prince GA, Jenson AB, Hemming VG, Murphy BR, Walsh EE, Horswood RL, Chanock RM. Enhancement of respiratory syncytial virus pulmonary pathology in cotton rats by prior intramuscular inoculation of formalin-inactivated virus. *J Virol*. 1986; 57:721–8. [PubMed: 2419587]
33. 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; 82:2881–8. [PubMed: 11714962]

34. Anderson LJ, Hierholzer JC, Bingham PG, Stone YO. Microneutralization test for respiratory syncytial virus based on an enzyme immunoassay. *J Clin Microbiol.* 1985; 22:1050–2. [PubMed: 4066916]
35. Anderson LJ, Bingham P, Hierholzer JC. Neutralization of respiratory syncytial virus by individual and mixtures of F and G protein monoclonal antibodies. *J Virol.* 1988; 62:4232–8. [PubMed: 2459412]
36. Gorman WL, Gill DS, Scroggs RA, Portner A. The hemagglutinin-neuraminidase glycoproteins of human parainfluenza virus type 1 and sendai virus have high structure-function similarity with limited antigenic cross-reactivity. *Virology.* 1990; 175:211–23. [PubMed: 1689918]
37. Brandenburg AH, Groen J, van Steensel-Moll HA, Claas EC, Rothbarth PH, Neijens HJ, Osterhaus AD. Respiratory syncytial virus specific serum antibodies in infants under six months of age: limited serological response upon infection. *J Med Virol.* 1997; 52:97–104. [PubMed: 9131465]
38. Feltes TF, Cabalka AK, Meissner HC, Piazza FM, Carlin DA, Top FH Jr, Connor EM, Sondheimer HM. Palivizumab prophylaxis reduces hospitalization due to respiratory syncytial virus in young children with hemodynamically significant congenital heart disease. *J Pediatr.* 2003; 143:532–40. [PubMed: 14571236]
39. Johnson S, Griego SD, Pfarr DS, Doyle ML, Woods R, Carlin D, Prince GA, Koenig S, Young JF, Dillon SB. A direct comparison of the activities of two humanized respiratory syncytial virus monoclonal antibodies: MEDI-493 and RSHZ19. *J Infect Dis.* 1999; 180:35–40. [PubMed: 10353858]
40. Meguro H, Kervina M, Wright PF. Antibody-dependent cell-mediated cytotoxicity against cells infected with respiratory syncytial virus: characterization of in vitro and in vivo properties. *J Immunol.* 1979; 122:2521–6. [PubMed: 571890]
41. Rudraraju R, Surman S, Jones B, Sealy R, Woodland DL, Hurwitz JL. Phenotypes and functions of persistent Sendai virus-induced antibody forming cells and CD8+ T cells in diffuse nasal-associated lymphoid tissue typify lymphocyte responses of the gut. *Virology.* 2011; 410:429–36. [PubMed: 21227475]
42. Dave VP, Allan JE, Slobod KS, Smith SF, Ryan K, Powell U, Portner A, Hurwitz JL. Viral cross-reactivity and antigenic determinants recognized by human parainfluenza virus type 1-specific cytotoxic T-cells. *Virology.* 1994; 199:376–83. [PubMed: 7510085]
43. Rudraraju R, Surman SL, Jones BG, Sealy R, Woodland DL, Hurwitz JL. Reduced frequencies and heightened CD103 expression among virus-induced CD8(+) T cells in the respiratory tract airways of vitamin A-deficient mice. *Clin Vaccine Immunol.* 2012; 19:757–65. [PubMed: 22398245]
44. Smith FS, Portner A, Leggiadro RJ, Turner EV, Hurwitz JL. Age-related development of human memory T-helper and B-cell responses toward parainfluenza virus type-1. *Virology.* 1994; 205:453–61. [PubMed: 7975247]
45. Koff WC, Caplan FR, Case S, Halstead SB. Cell-mediated immune response to respiratory syncytial virus infection in owl monkeys. *Clin Exp Immunol.* 1983; 53:272–80. [PubMed: 6883807]
46. Sato H, Albrecht P, Reynolds DW, Stagno S, Ennis FA. Transfer of measles, mumps, and rubella antibodies from mother to infant. Its effect on measles, mumps, and rubella immunization. *Am J Dis Child.* 1979; 133:1240–3. [PubMed: 229727]
47. Kim D, Huey D, Oglesbee M, Niewiesk S. Insights into the regulatory mechanism controlling the inhibition of vaccine-induced seroconversion by maternal antibodies. *Blood.* 2011; 117:6143–51. [PubMed: 21357766]
48. Van de Perre P. Transfer of antibody via mother's milk. *Vaccine.* 2003; 21:3374–6. [PubMed: 12850343]
49. Prince GA, Horswood RL, Chanock RM. Quantitative aspects of passive immunity to respiratory syncytial virus infection in infant cotton rats. *J Virol.* 1985; 55:517–20. [PubMed: 4020957]
50. De Vincenzo JP, Leombruno D, Soiffer RJ, Siber GR. Immunotherapy of respiratory syncytial virus pneumonia following bone marrow transplantation. *Bone Marrow Transplant.* 1996; 17:1051–6. [PubMed: 8807113]
51. Malley R, DeVincenzo J, Ramilo O, Dennehy PH, Meissner HC, Gruber WC, Sanchez PJ, Jafri H, Balsley J, Carlin D, Buckingham S, Vernacchio L, Ambrosino DM. Reduction of respiratory

- syncytial virus (RSV) in tracheal aspirates in intubated infants by use of humanized monoclonal antibody to RSV F protein. *J Infect Dis.* 1998; 178:1555–61. [PubMed: 9815203]
52. Niewiesk S. Studying experimental measles virus vaccines in the presence of maternal antibodies in the cotton rat model (*Sigmodon hispidus*). *Vaccine.* 2001; 19:2250–3. [PubMed: 11257342]
 53. Blomqvist GA, Lovgren-Bengtsson K, Morein B. Influence of maternal immunity on antibody and T-cell response in mice. *Vaccine.* 2003; 21:2022–31. [PubMed: 12706692]
 54. Sami IR, Piazza FM, Johnson SA, Darnell ME, Ottolini MG, Hemming VG, Prince GA. Systemic immunoprophylaxis of nasal respiratory syncytial virus infection in cotton rats. *J Infect Dis.* 1995; 171:440–3. [PubMed: 7844385]
 55. Johnson S, Oliver C, Prince GA, Hemming VG, Pfarr DS, Wang SC, Dormitzer M, O'Grady J, Koenig S, Tamura JK, Woods R, Bansal G, Couchenour D, Tsao E, Hall WC, Young JF. Development of a humanized monoclonal antibody (MEDI-493) with potent in vitro and in vivo activity against respiratory syncytial virus. *J Infect Dis.* 1997; 176:1215–24. [PubMed: 9359721]
 56. 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; 121:23–32. [PubMed: 16677733]
 57. Sealy R, Jones BG, Surman SL, Hurwitz JL. Robust IgA and IgG-producing antibody forming cells in the diffuse-NALT and lungs of Sendai virus-vaccinated cotton rats associate with rapid protection against human parainfluenza virus-type 1. *Vaccine.* 2010; 28:6749–56. [PubMed: 20682364]
 58. 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; 171:4969–73. [PubMed: 14607890]

Highlights

- SeVRSV induces RSV-specific antibodies in a maternal antibody cotton rat model
- SeVRSV protects against RSV challenge in a maternal antibody cotton rat model
- Polyclonal or monoclonal, SeV- or RSV-specific antibodies fail to inhibit SeVRSV efficacy

Figure 1. Maternal antibody model in cotton rats

The strategy for testing SeVRSV vaccine efficacy in the presence of passively transferred neutralizing antibodies is illustrated. Cotton rats were first passively transferred with neutralizing antibodies from various sources. Two days later, blood samples were tested and animals were immunized with SeVRSV. Three months later, cotton rats were tested for post-vaccine antibody neutralization titers and challenged with RSV. Lungs were harvested 3–4 days after challenge to determine if SeVRSV had successfully protected animals from RSV infections.

Figure 2. Loss of passively transferred antibodies over time

Sera were collected from cotton rats at various time points after RespiGam (2 ml, 100 mg, IP) injections. These animals were not vaccinated. An ELISA with specificity for human antibodies and no cross-reactivity with cotton rat antibodies was used to monitor the loss of the passively transferred human antibodies over time. A. Sera were diluted 1:1,000 and examined on days 0, 2, 5, 7, 14 and 21 after RespiGam injections to monitor antibody loss. Results are shown for 3 test animals. Controls included diluted RespiGam (positive control, 1:10,000 dilution) and sera from cotton rats (CR) that had or had not been immunized with SeVRSV ('CR sera-unvacc' and 'CR sera-vacc' negative controls). B. Sera were examined 3 months after RespiGam injections in the three test cotton rats, at which time the passively transferred antibodies could no longer be detected.

Figure 3. Passively transferred antibodies fail to protect against RSV three months after injection

Cotton rats received passive antibody transfers with RespiGam. Control animals received no antibodies. Cotton rats were not immunized with SeVRSV in these experiments. Three months after passive antibody transfers, cotton rats were challenged with RSV. Three days later, animals were sacrificed to measure RSV titers in cotton rat lungs. Each point represents a different animal, demonstrating that the passively transferred antibodies were not protective after a three month rest period.

A.

Animal #	Respi-Gam Antibody	Pre-Vaccine SeV Neut. Titer	Pre-Vaccine RSV Neut. Titer	Vaccine	Post-Vaccine SeV Neut. Titer	Post-Vaccine RSV Neut. Titer
1	None	<40	<40	None	<40	<40
2	None	<40	<40	None	ND	ND
3	None	<40	<40	None	<40	<40
4	None	<40	<40	None	<40	<40
5	None	<40	<40	SeVRSV	1280	160
6	None	<40	<40	SeVRSV	>2560	160
7	None	ND	<40	SeVRSV	>2560	320
8	1 mg	<40	40	SeVRSV	1280	320
9	1 mg	<40	80	SeVRSV	>2560	160
10	1 mg	<40	80	SeVRSV	640	160
11	1 mg	<40	40	SeVRSV	1280	160
12	10 mg	<40	640	SeVRSV	>2560	80
13	10 mg	<40	640	SeVRSV	320	160
14	10 mg	<40	1280	SeVRSV	640	40

B.

Figure 4. SeVRSV protects cotton rats from RSV challenge in the presence of polyclonal RespiGam

A. Results of SeV and RSV neutralization assays with sera taken before vaccination or three months after vaccination are shown. ND=not determined.

B. Viral titers are plotted from lungs taken after RSV challenge. Each symbol represents a different animal.

Statistical analyses were conducted using the Fishers Exact Test. Protection from RSV challenge was considered positive if RSV titers scored below 1×10^4 PFU. When test animals were vaccinated after having received the highest dose of RespiGam (10 mg), titers were significantly reduced compared to those of untreated, unvaccinated controls ($p < .05$).

A.

Animal Number	GAMUNEX	Pre-Vaccine SeV Neut. Titer	Pre-Vaccine RSV Neut. Titer	Vaccine	Post-Vaccine RSV Neut. Titer
1	None	<40	<40	None	<40
2	None	<40	<40	None	40
3	None	<40	<40	None	40
4	None	<40	<40	SeVRSV	1280
5	None	<40	40	SeVRSV	1280
6	None	<40	<40	SeVRSV	1280
7	None	<40	<40	SeVRSV	1280
8	10 mg	<40	320	SeVRSV	320
9	10 mg	40	640	SeVRSV	320
10	10 mg	40	320	SeVRSV	160
11	10 mg	<40	40	SeVRSV	>2560
12	25 mg	40	1280	SeVRSV	<40
13	25 mg	<40	40	SeVRSV	1280
14	25 mg	40	1280	SeVRSV	160
15	25 mg	40	1280	SeVRSV	160
16	100 mg	160	>2560	SeVRSV	40
17	100 mg	160	>2560	SeVRSV	<40
18	100 mg	160	>2560	SeVRSV	<40
19	100 mg	80	>2560	SeVRSV	<40

B.

Figure 5. SeVRSV protects cotton rats from RSV challenge in the presence of polyclonal GAMUNEX

Animals were passively transferred with GAMUNEX 2 days prior to vaccination with SeVRSV.

A. Results of SeV and RSV neutralization assays with sera taken before vaccination or three months after vaccination are shown.

B. Viral titers are plotted from lungs taken after RSV challenge. Each symbol represents a different animal.

Statistical analyses were conducted using the Fishers Exact Test. Protection from RSV challenge was considered positive if RSV titers scored below 1×10^4 PFU. When test animals were vaccinated after having received the highest dose of GAMUNEX (100 mg), titers were significantly reduced compared to those of untreated, unvaccinated controls ($p < .05$).

Figure 6. SeVRSV is replication-competent in the context of passively transferred antibodies
 To determine if SeVRSV remained replication-competent in the context of passively transferred antibodies, animals were injected with GAMUNEX and vaccinated with SeVRSV 2 days later. Four days after vaccination, cotton rat lungs were tested for replication-competent SeVRSV. Unvaccinated, control animals scored negatively for virus. Each symbol represents the virus titer from an individual cotton rat in vaccinated groups.

A.

Animal Number	SeV-specific Antibody	Pre-Vaccine SeV Neut. Titer	Pre-Vaccine RSV Neut. Titer	Vaccine	Post-Vaccine RSV Neut. Titer
1	None	<40	<40	None	<40
2	None	<40	<40	None	<40
3	None	<40	<40	None	<40
4	None	<40	<40	None	<40
5	0.36 mg	160	<40	None	<40
6	0.36 mg	320	<40	None	<40
7	0.36 mg	160	<40	None	<40
8	None	<40	<40	SeVRSV	320
9	None	<40	<40	SeVRSV	320
10	None	<40	<40	SeVRSV	320
11	None	<40	<40	SeVRSV	80
12	0.12 mg	160	<40	SeVRSV	1280
13	0.12 mg	160	<40	SeVRSV	320
14	0.12 mg	80	<40	SeVRSV	80
15	0.12 mg	40	<40	SeVRSV	160
16	0.36 mg	320	<40	SeVRSV	320
17	0.36 mg	320	<40	SeVRSV	320
18	0.36 mg	320	<40	SeVRSV	80
19	0.36 mg	320	<40	SeVRSV	320

B.

Figure 7. SeVRSV protects cotton rats from RSV challenge in the presence of monoclonal SeV-specific antibodies

Animals were passively transferred with two SeV-specific monoclonal antibodies two days prior to vaccination with SeVRSV.

A. Results of SeV and RSV neutralization assays with sera taken before vaccination or three months after vaccination are shown.

B. Viral titers are plotted from lungs taken after RSV challenge. Each symbol represents a different animal.

Statistical analyses were conducted using the Fishers Exact Test. Protection from RSV challenge was considered positive if RSV titers scored below 1×10^4 PFU. When test animals were vaccinated after having received the highest dose of SeV-specific monoclonal antibodies (.36 mg), titers were significantly reduced compared to those of untreated, unvaccinated controls ($p < .05$).

Figure 8. SeVRSV protects cotton rats from RSV challenge in the presence of RSV F-specific antibodies

Animals were passively transferred with Palivizumab two days prior to vaccination with SeVRSV.

A. Results of SeV and RSV neutralization assays with sera taken before vaccination or three months after vaccination are shown. Our previous experiments demonstrated that, as expected, Palivizumab provided no neutralizing activity toward SeV.

B. Viral titers are plotted from lungs taken after RSV challenge. Each symbol represents a different animal.

Statistical analyses were conducted using the Fishers Exact Test. Protection from RSV challenge was considered positive if RSV titers scored below 1×10^4 PFU. When test animals were vaccinated after having received the highest dose of Palivizumab (10 mg), titers were significantly reduced compared to those of untreated, unvaccinated controls ($p < .05$).

TABLE 1

SeV and RSV-specific neutralizing antibodies decrease with age in young infants

0 to 2 month age group [#]			2 to 4 month age group			4 to 6 month age group		
Age in Days	SeV neut	RSV neut	Age in Days	SeV neut	RSV neut	Age in Days	SeV neut	RSV neut
4	320	640	62	<40	80	124	<40	40
5	320	160	*62	<40	80	*127	40	40
5	160	320	64	<40	160	*134	<40	40
7	80	160	69	80	<40	137	80	160
7	80	160	76	40	80	142	40	40
14	320	80	*76	<40	80	145	40	<40
*19	80	160	77	40	160	153	<40	<40
25	160	320	77	40	160	153	<40	160
26	80	320	88	80	160	154	<40	40
*32	80	40	*90	<40	40	156	160	<40
36	<40	40	*94	<40	80	158	40	160
*39	<40	80	96	40	160	165	<40	<40
*43	80	80	104	<40	40	*168	40	<40
47	40	160	*118	<40	80	170	<40	40
Geometric Mean	69	113		30	80		38	38
Range	<40–2560	<40–640		<40–80	<40–160		<40–160	<40–160

[#] Samples (n=33, 31, and 23 from age groups 0 to 2 months (4–61 days), 2 to 4 months (62–122 days), and 4 to 6 months (123–183 days), respectively) were tested for neutralization activities. Results from 14 representative samples in each group are shown. A minority of samples from the younger age groups were tested for SeV neutralization only, due to limited sample volume. Samples are identified by age of patient in days. Geometric means and ranges are shown for the full data set. To calculate geometric means, values of <40 were assigned a numerical value of 20.

* Individuals with RSV infections were diagnosed by PCR. Control adult sera scored with a range of titers from 160 to >2560 in the RSV neutralization assay and <40 to >640 in the SeV neutralization assay.

TABLE 2

RSV infection associates with reduced RSV neutralization serum titers

Patient Group	RSV infected*					Other (symptomatic and non-symptomatic)				Total samples tested
	RSV neutralization titers					RSV neutralization titers				
	0 to 2 mo.	2 to 4 mo.	4 to 6 mo.	Sub total		0 to 2 mo.	2 to 4 mo.	4 to 6 mo.	Sub total	
Age group										
Titer>100	2	1	0	3		12	10	3	25	28
Titer<100	6	7	6	19		6	12	14	32	51
Total	8	8	6	22		18	22	17	57	79

Statistical comparison between 'RSV infected' and 'Other' by Fishers Exact: p<.02

Within age groups 0 to 2 months, 2 to 4 months, and 4 to 6 months, there were 8, 8, and 6 'RSV infected' patients and 18, 22, and 17 'other' patients, respectively, for whom blood samples were available for RSV neutralization assays (a minority of collected samples were not tested for RSV serum neutralization titers due to limited sample volumes).

* RSV infection was defined as clinical evidence of acute respiratory disease and detection of RSV in their respiratory secretions by Real Time RT-PCR. Patients who were not positive for RSV were either those who were hospitalized for respiratory symptoms but whose secretions tested negative for RSV by Real Time RT-PCR, or who were asymptomatic for respiratory illnesses. The number of samples with titers >100 or <100 among 'RSV infected' and 'Other' patients were tabulated and compared with a Fishers Exact Test.