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Maternal transfer of RSV immunity in cotton rats vaccinated during pregnancy

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ABSTRACT

Respiratory Syncytial Virus (RSV) is the leading cause of pneumonia and bronchiolitis in infants, resulting in significant morbidity and mortality worldwide. There is currently no RSV vaccine. Although maternal serum antibodies against RSV are efficiently transferred through placenta protecting human infants from RSV-induced disease, this protection is short-lived and the methods for extending and augmenting protection are not known. The objective of this study was to develop an animal model of maternal RSV vaccination using the *Sigmodon hispidus* cotton rat. Naïve or RSV-primed female cotton rats were inoculated with live RSV and set in breeding pairs. Antibody transfer to the litters was quantified and the offspring were challenged with RSV at different ages for analysis of protection against viral replication and lung inflammation. There was a strong correlation between RSV-neutralizing antibody (NA) titers in cotton rat mothers and their pups, which also correlated with protection of litters against virus challenge. Passive protection was short-lived and strongly reduced in animals at 4 weeks after birth. Protection of litters was significantly enhanced by inoculating mothers parenterally with live RSV and inversely correlated with the expression of lung cytokines and pathology. Importantly, vaccination and boosting of naïve mothers with the live RSV produced the highest levels of NAs. We conclude that maternal vaccination against RSV in the cotton rat can be used to define vaccine preparations that could improve preexistent immunity and induce subsequent transfer of efficient immunity to infants.

1. Introduction

Respiratory Syncytial Virus (RSV) is the leading cause of pneumonia and bronchiolitis in infants, resulting in significant morbidity and mortality worldwide [1]. There is no vaccine approved for RSV infection. A vaccine for future mothers that can extend passive protection of babies in the critical period between 2 and 6 months after birth and beyond would be highly desirable [2]. In addition, such a vaccine could have a secondary protective effect in the household by preventing spread of RSV within families [3]. In an era when maternal vaccination is increasingly considered to be a valuable strategy to protect both the mother and infant against RSV, further research is needed to evaluate the potential beneficial effect of different vaccine formulations. However, characterization of maternal vaccines could be complicated since transfer of immunity from mother to infant is dependent on a large range of complex variables that need to be defined in a preclinical model of RSV vaccination.

Antibodies against RSV are found universally in adult sera [4], and pooled adult sera have been shown to be protective in high-risk infants against RSV infection and disease [5]. In addition, studies have shown evidence that the higher the levels of maternal anti-RSV neutralizing antibody (NA) titers, the longer the protection of the babies. It was reported that the severity of RSV disease in infants <9 months old was reduced when levels of maternal antibodies were higher [6] and that higher levels of antibodies at the time of birth correlated with later times of infant infection [7].

Maternal immunization has shown efficacy for influenza, tetanus, and pertussis [2,8] and this vaccination regimen is particularly convenient and effective in resource-limited settings. In babies, there is a selective transfer of antibody isotypes from mothers, with the IgG1 subtype predominating [9]. In addition, maternal vaccination has been shown to be safe for both mother and infants. For example, vaccination of pregnant women against influenza during all trimesters is now widely used in the U.S.A. and has not been

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associated with increased risk of preterm or small gestational age births [10].

The cotton rat model is extensively used for testing RSV vaccines and therapeutics. The strength of this model for studying passive immunity has been well recognized as evidenced by the development of the antibody-based prophylactic therapies (*i.e.*, RespiGam® and Synagis®) against RSV [11,12]. Previous studies demonstrated that immune cotton rats transfer maternal immunity through placenta and by nursing [13]. In this work, we characterize the parameters and conditions that will be important for optimizing vaccines that will enhance maternal transfer of immunity to RSV. Study of different vaccination strategies could identify one that selectively enhances natural mechanisms of the transfer of maternal immunity and extend the period of protection. This work establishes the basis for a model that attempts to reflect maternal immunization and passive transfer of immunity in humans with the expectation that it will permit us ultimately to enhance maternal immunity and to develop protection against RSV in the older infants and children.

2. Materials and methods

2.1. Animals

Inbred *Sigmodon hispidus* cotton rats were obtained from a colony maintained at Sigmovir Biosystems, Inc. (Rockville, MD). Three to five-week-old female animals were used for vaccination experiments. Animals were pre-bled before being included in the study to rule out the possibility of preexistent antibodies against RSV. Animals were housed in large polycarbonate cages and fed a standard diet of rodent chow and water *ad libitum*. The colony was monitored for antibodies to paramyxoviruses and rodent viruses, and no such antibodies were found. All studies were conducted under applicable laws and guidelines and after approval from the Sigmovir Biosystems, Inc. Institutional Animal Care and Use Committee.

2.2. Viruses and viral assays

The prototype Long strain of RSV was obtained from American Type Culture Collection (ATCC VR-26, Manassas, VA). Virus was propagated in HEp-2 cells and serially plaque-purified to reduce defective-interfering particles. The single pool of virus containing $10^{7.6}$ pfu/ml was used for all experiments. To adjust the dose, stock was diluted with PBS for intranasal (i.n.) and intramuscular (i.m.) immunization. Viral titers in the lungs and in the nose of RSV-infected infant cotton rats were determined as described elsewhere [14] and adjusted by the weight of the lung portion or expressed per nose.

2.3. RSV neutralizing antibody (NA) assay

RSV NA titers were measured by 60% plaque reduction assay using four-fold dilutions of heat-inactivated serum samples against the RSV/A/Long strain (25–50 pfu) in Hep-2 cells incubated in 24-well plates at 37 °C after overlaying the wells with 0.75% methylcellulose medium. After 4 days of incubation, the overlay was removed and the cells were fixed in 2.5% glutaraldehyde solution containing 0.1% crystal violet for 1 h, rinsed, and air-dried. The corresponding reciprocal NA titers were determined as previously indicated [15]. The limit of detection (LOD) of this assay was 4.32 Log_2 or a 1:20 dilution of the serum [14].

2.4. Cytokine expression by Real-time PCR

Total RNA was extracted from homogenized lung tissue using the RNeasy purification kit (QIAGEN). One microgram of total RNA was used to prepare cDNA in a volume of 20 μl (QuantiTect Reverse Transcription Kit from Qiagen). cDNA was diluted to 10 μg/ml and 3 μl were used for each 25 μl real-time PCR reaction (QuantiFast SYBR Green PCR Kit from Qiagen) with final primer concentrations of 0.5 μM. Reactions were set up in 96-well plates and amplifications were performed on a Bio-Rad iCycler (MyiQ Single Color). Delta *Ct* method was used to calculate relative gene expressions, that were normalized to β-actin as a housekeeping gene [16].

2.5. Lung histopathology

Lungs were dissected and inflated with 10% neutral buffered formalin to their normal volume, and then immersed in the same fixative solution. Following fixation, lungs were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). An average total score was determined for each group based in four parameters of pulmonary inflammation: peribronchiolitis (inflammatory cell infiltration around the bronchioles), perivasculitis (inflammatory cell infiltration around the small blood vessels), interstitial pneumonia (inflammatory cell infiltration and thickening of alveolar walls), and alveolitis (cells within the alveolar spaces). Slides were scored blindly on a 0–4 severity scale as previously described [16].

2.6. Experimental design

During the first experiment (outlined in Fig. 1A), two groups of female cotton rats (3–5 weeks of age; 10 rats/group) were "primed" by intranasal (i.n.) or intramuscular (i.m.) inoculation with live RSV (10^5 PFU/animal). A third group of 5 females remained untreated as controls (unprimed). Two weeks post-priming, all females were mated in separate cages with naïve, age-matched males. Before delivery, all females were bled for the determination of total anti-RSV NA. Each litter was allocated randomly to one of 4 different subgroups, corresponding to pups challenged at 1, 2, 3, and 4 weeks after birth. Each subgroup had 12 to 22 cotton rat pups from at least two different mothers. To prevent pups from being rejected by the mother and to keep 3–4 week age groups at the same nursing level, all pups reaching age 21 days were weaned. Each subgroup of pups was challenged under isoflurane anesthesia with RSV/A/Long (10^5 pfu/pup) using a corrected volume for intranasal inoculation: 1- and 2-week pups with 25 μl, 3-week pups with 40 μl, and 4-week pups with 50 μl. Pups were bled and sacrificed at day 4 *p.i.* The left lobe of the lung and the noses were homogenized in 1 ml of HBSS +10%SPG +1% Fungizone +0.1% Gentamicin for the determination of the nose and lung viral titers as previously described [14].

In a second experiment (outlined in Fig. 2A), 25 female cotton rats, 3-weeks old, were separated into 5 groups. Animals in Group A remained naïve throughout experiment and gave birth to naive pups that were subsequently challenged with RSV. Animals in Groups B, C, and D were "primed" by i.n. infection with RSV/A/Long (10^5 pfu/100 μl/rat) on day 0. Two weeks later, animals in Groups C and D were vaccinated i.m. with live RSV/A/Long (10^5 pfu). Animals in Group E were not primed, but were vaccinated i.m. with live RSV/A/Long (10^5 pfu) at the same time as Groups C and D. At week 5, all cotton rats in each group were separated into single cages and paired with a naïve male cotton rat. On week 7, pregnant females in Groups D and E, were boosted i.m. with the same live RSV preparation. Serum samples from all females were obtained after the first vaccination (week 6), after the boost but before delivery (week 8), and again, before the second delivery (week 20). The