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Maternal immunization with a recombinant adenovirus-expressing fusion protein protects neonatal cotton rats from respiratory syncytia virus infection by transferring antibodies *via* breast milk and placenta

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ABSTRACT

We evaluated the efficacy of a recombinant adenovirus that expresses a membrane-truncated respiratory syncytial virus (RSV) fusion protein (Ad-F0ΔTM) in newborns *via* maternal immunization (MI) of pregnant cotton rats. Intranasal Ad-F0ΔTM immunization was given to pregnant female rats, and MI-newborn rats were then challenged intranasally with RSV. Anti-RSV IgGs were observed in the serum of MI-newborn rats after birth. The pulmonary viral loads in Ad-F0ΔTM *vs.* control vector, Ad-LacZ, and MI-newborns on day 3 post-challenge were reduced by 4 $\log_{10}$/g lung. The neutralizing antibody remained for up to 3 weeks in the serum of MI-newborns, which is when weaning began. Ad-F0ΔTM protected MI-newborns from RSV challenge for 1 week. Vertical-transferred protective antibodies were examined in the breast milk and placenta as well. Finally, anti-RSV immunity was not boosted but was only primed during the next RSV exposure in Ad-F0ΔTM-MI-newborns. Maternal Ad-F0ΔTM immunization provides acute protection against RSV infection in neonates.

1. Introduction

Respiratory syncytial virus (RSV) is the most common cause of respiratory tract inflammatory diseases, such as bronchiolitis and pneumonia, and is associated with profound morbidity and mortality in premature babies and infants (Borchers et al., 2013; Chanock, 1957; Falsey, 2005; Holzel et al., 1963). RSV can also become serious in older adults or anyone with an immunocompromised condition (Ebbert and Limper, 2005).

The only approach for RSV prophylaxis is *via* passive immunization with palivizumab (brand name Synagis®), which is a neutralizing antibody that targets RSV F protein; it has been the most successful approach but has limited use in high-risk infants and children (Resch, 2014; Vissers et al., 2015; Weltzin and Monath, 1999). In a 1960s clinical trial, infants received formalin-inactivated RSV (FI-RSV); however, the poor neutralizing antibodies formed immune complexes with RSV and resulted in the enhancement of diseases (Delgado et al., 2009; Polack et al., 2002). Consequently, there has been a large emphasis on the safety of any proposed RSV vaccines that protect newborn infants. Therefore, approaches including the immunization of pregnant mothers require high safety margins. Our immune response to natural RSV infection is very short-lived, allowing multiple infections annually (Groothuis and Simoes, 1993). Moreover, immunization with live attenuated vaccines has been difficult due to having to identify an optimal balance between virus replication and non-vaccine-enhanced symptoms (Wright et al., 2007). These are challenges to any new vaccine candidate to protect against RSV.

The infection of a permissive cell line with a recombinant replication-defective adenoviral vector (Ad)-expressing antigen as a vaccine has been developed and tested in multiple successful preclinical studies (Coughlan et al., 2015; Kohlmann et al., 2009; Shao et al., 2009). Ads are highly immunogenic. They result in the apoptotic death of the infected cells, as well as mature macrophages and dendritic cells. Both cell types respond to Ads by producing cytokines, such as IL-6, IL-12, and tumor necrosis factor-α (TNF-α) (Morelli et al., 2000; Zhang et al., 2001), and serve as antigen-presenting cells that subsequently stimulate adaptive immune effectors, T helper cells and cytotoxic T-cell responses (Palucka and Banchereau, 2002). Activation of the B-cell response to

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secrete transgene product-specific antibodies is mainly of the IgG2a isotype but also IgG1 in mice, suggesting a mixed Th1/Th2 response that predominated towards the Th1-type (Tatsis and Ertl, 2004). Ads with a natural tropism for the respiratory epithelium have been proven displaying low virulence and superior gene transfer efficiency (Hitt and Graham, 2000), making them convenient for immunizations against respiratory pathogens (Coughlan et al., 2015; Shao et al., 2009; Wang et al., 2004).

The cotton rat has previously been used as a model to understand the outcomes of maternal vaccination against RSV. The anti-RSV neutralizing antibodies, which are vertically transferred from female rats primed with live RSV and are then set in breeding pairs, were strongly correlated with the protection of offspring against RSV challenge and were inversely correlated with pulmonary cytokine secretions and pathology (Blanco et al., 2015). We previously created an E1/E3-deleted Ad5 genome that carried the transmembrane-deleted fusion (F) gene of the RSV (F0ΔTM). The immunogenicity of Ad-F0ΔTM, which was tested in mice (Shao et al., 2009) and cotton rats (Shao et al., 2016), showed that anti-F neutralizing antibody production, as well as Th1/Th2-balanced cellular immunity, were induced by the prime and boost but not by a single dose of intranasal (i.n.) immunization (Shao et al., 2009). Moreover, Ad vaccine-enhanced pulmonary diseases were not observed, indicating that Ad-F0ΔTM immunization was reliable in these animals. Several studies have shown that the passive transfer of protective antibodies to newborns was achieved by maternal immunization in both animal and human studies (Blanco et al., 2015; Jones et al., 2014; Sharma et al., 2014; Zaman et al., 2008). Here, we studied the efficacy of Ad-F0ΔTM in a cotton rat model by giving pregnant mothers maternal immunizations and then by challenging the newborn rats with RSV. We demonstrated that neutralizing anti-RSV antibodies induced by the Ad-F0ΔTM vaccine were raised in the mother, were transferred to the newborn and then remained in the newborn for one week to protect against RSV challenge. In addition to placental transfer, the anti-RSV antibodies in the blood of newborns were mostly transferred from the breast milk of Ad vaccine-immunized pregnant rats. These results support the clinical use of the Ad-RSV vaccine in future populations.

2. Materials and methods

2.1. Ethics statement

We confirm that all the methods used in the animal experiments were performed in accordance with the guidelines of the Laboratory Animal Center of the National Health Research Institutes (NHRI), Taiwan (http://lac.nhri.org.tw/e_index.php.). The animal use protocols were reviewed and approved by the National Health Research Institutes (NHRI) Institutional Animal Care and Use Committee (Approval Protocol No. NHRI-IACUC-103052A). To perform the immunization or the viral challenge, the animals were placed in an anesthetic inhalational chamber containing isoflurane (initial phase - 5% and maintenance phase - 1.5–2.5%) for 1 min before intranasal Ad-vaccine immunization. After investigation, the test animals were euthanized by 100% CO_2 inhalation for 5 min.

2.2. Cell lines and viruses

Human larynx carcinoma cells (Hep-2; originally purchased from the American Type Culture Collection, ATCC No. CCL-23) were obtained from Dr. Barney S. Graham at the Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, USA, and the human embryonic kidney cells (293 A) were purchased from Invitrogen-Thermo Fisher Scientific Inc., CA, USA (Cat. R70507). The cells were grown and maintained in DMEM (GE Healthcare HyClone, UT, USA, Cat. SH300) supplemented with 10% FBS and 1% penicillin/streptomycin (Biological) in an incubator maintained at 37 °C and were equilibrated with 5% CO_2. The human RSV-B1 strain from ATCC was propagated in Hep-2 cells (Shao et al., 2009). Viral stocks were stored at −80 °C. Virus stock titers were tested with a standard plaque-forming assay (Lin et al., 2012), and the number of plaque-forming units (pfus) were calculated.

An E1/E3-deleted Ad5 genome that encoded the transmembrane-deleted F gene of the RSV to have Ad-F0ΔTM, or the reporter gene of bacterial β-galactosidase (LacZ) to gain Ad-LacZ, were propagated in HEK-293A cells that were cultivated in the presence of 10% FBS (Shao et al., 2009). The recombinant Ads were purified and concentrated using a Vivapure adenoPACK 100RT (Satorius Stedin Biotech, Goettingen, German). The purified virus titer was determined using a modified standard plaque assay (Shao et al., 2009).

2.2.1. Maternal immunization and live RSV challenge

Six- to eight-week-old cotton rats (purchased from Sigmovir, LTD, USA) were maintained in pathogen-free cages at the Laboratory Animal Center at NHRI throughout the animal study. For maternal immunization with the Ad vaccine, pairs of cotton rats were mated on day 0; then, the female rats were i.n. primed and boosted with 5×10^7 and 1×10^8 pfu/100 μL, respectively, of Ad at 14-day intervals 7 days after mating. The newborn rats were usually birthed on day 28 after mating. The newborn rats were fed by their own mothers, and their serum was collected on day 6 after the day of birth (DOB), followed by an i.n. challenge with 4×10^6 pfu/75 μL of live RSV-B1 one day later. To switch the surrogacy to bleed the consanguinity of the maternal-immunized (MI) offspring, the Ad-F0ΔTM-MI neonates were bled immediately from Ad-LacZ-immunized mothers (called as AdF0ΔTM/WT milk) within 1-h post-DOB. In contrast, the Ad-LacZ-MI newborns were bled immediately from Ad-F0ΔTM-immunized mothers (called as WT CR/AdF0ΔTM immune milk) within 1-h post-DOB. Ad-LacZ-MI litters from Ad-LacZ-immunized mothers (Ad-LacZ/Ad-LacZ milk) that were bled were included as the control. Then, the MI newborns were challenged with 4×10^6 pfu/75 μL of live RSV on weeks 1 or 3 or with 4×10^6 pfu/200 μL of live RSV on week 5 post-DOB. Their serum was collected one day before challenge. The MI newborns were sacrificed on day 3 after the viral challenge, and the lung tissues were isolated for plaque assays. To collect whey samples from the Ad-MI mother rats, the mother rats were injected with 2 IU/rat of oxytocin (Sigma-Aldrich, Inc., USA, Cat. No. O3251) at day 0 post-birth, and then breast tissues were extruded to obtain secreted whey at days 7 and 10 post-birth.

The sera were individually analyzed against heat-inactivated (75 °C for 1 h) RSV-B1 (HI-RSV) or anti-Ad5 vector with an ELISA for virus-specific neutralizing activity. Whole lungs (0.1–0.2 g) were excised from the rats. For the plaque assay, the lungs placed in 200 μL of PBS were homogenized, clarified, and titrated by plaque assays on Hep-2 cells in 12-well plates. Then, the cells were inoculated with 100 μL per well of 10-fold serially diluted lung homogenates in serum-free DMEM for 1 h at 37 °C and 5% CO_2, were overlaid with 1.5% methylcellulose (Sigma-Aldrich, Inc., USA) in DMEM, and were then 10% heat-inactivated (56 °C for 30 min) with fetal bovine serum (HI-FBS; Biological) for 7 days. The cells were stained with hematoxylin and eosin (H/E), and the plaques were counted 1 day later. The assay limit of detection was 2 pfu/whole lung. The value = 0 was used for statistical analyses if the titer was below the limit of detection.

2.3. Enzyme-linked immunosorbent assay (ELISA)

To detect anti-RSV IgG and IgA antibodies in the sera or whey, 96-well plates were coated with 100 μL per well of 5×10^4 pfu HI-RSV in carbonate buffer (pH 8.0). Alternatively, Ad-LacZ immobilized on ELISA wells (2.5×10^4 pfu of the virus per well) using the same coating condition to assay the contents of anti-Ad antibodies in the sera were performed. The sera collected from the immunized rat were inactivated at 56 °C for 30 min. Two-fold serial dilutions of HI-sera were performed by adding normal cotton rat sera, beginning from a vary-fold initial