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The respiratory syncytial virus fusion protein formulated with a novel combination adjuvant induces balanced immune responses in lambs with maternal antibodies

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ABSTRACT

Respiratory syncytial virus (RSV) causes severe respiratory illness in infants. There are no licensed vaccines to prevent RSV infection. The neonate receives short-term protection from maternally derived antibodies, which, however, can also interfere with the active response to vaccination. A RSV vaccine consisting of a truncated version of the fusion protein formulated with polyI:C, innate defense regulator peptide and polyphosphazene (ΔF/TriAdj), was evaluated in two to three week-old lambs. When delivered intrapulmonary, ΔF/TriAdj elicited IgA production in the lung in addition to a robust systemic response similar to that induced by intramuscular immunization. To investigate potential interference by maternal antibodies, pregnant ewes were vaccinated with ΔF/TriAdj. Lambs born to RSV F-immune or non-immune ewes were then given three vaccinations with ΔF/TriAdj at 3 days, 4 weeks and 8 weeks post-birth. Lambs immunized intramuscularly with ΔF/TriAdj vaccine developed high-affinity ΔF-specific serum IgG and virus neutralizing antibodies, and displayed an increase in the frequency of IFN-γ-secreting cells by *in vitro* restimulated peripheral blood mononuclear cells. Maternal antibodies did not interfere with the development of an immune response to ΔF/TriAdj in the newborn lambs. These results indicate that immunization of neonates with ΔF/TriAdj is effective even in the face of maternal antibodies.

1. Introduction

Respiratory syncytial virus (RSV) is a major cause of respiratory tract illness in infants and children leading to hospitalization and thousands of deaths each year worldwide [1]. The successful development of a RSV vaccine poses many challenges such as immature immune responses of infants, the potential interference of maternal antibodies with vaccination, and earlier concerns of vaccine-induced enhanced disease severity. In the 1960s, a formalin-inactivated RSV (FI-RSV) vaccine failed to induce RSV-specific neutralizing antibodies and unexpectedly caused vaccine-enhanced disease after natural RSV infection [2]. Many vaccine candidates, including live attenuated strains, as well as vector-based and subunit protein vaccines, have been evaluated in rodent and primate models, but there are still no safe and effective RSV vaccines or specific treatments other than prophylaxis with passive antibody therapy (Palivizumab) [3].

The RSV fusion protein (F) is highly conserved and facilitates penetration of the virus into the host cell and subsequent formation of syncytia thus making it a suitable subunit vaccine candidate [4]. It has been shown that immunization with purified F protein alone is insufficient to induce protection due to poor immunogenicity [5,6]. Thus, the F protein needs to be formulated with an adjuvant to induce robust immune responses including high-affinity neutralizing antibodies. Previously, we have demonstrated the induction of protective immunity by vaccination with a novel RSV vaccine candidate consisting of a truncated fusion (ΔF) protein formulated with a TLR3 agonist (polyI:C), an immune defense regulator (IDR) peptide and a synthetic polymer, polyphosphazene (ΔF/TriAdj) in rodents [6,7]. Recently, we also revealed that the duration of protective immunity induced with this vaccine is at least one year [8].

Lamb models have been increasingly used to study RSV pathogenesis, immune responses, vaccination and therapeutic strategies

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as they have similar lung development and mucosal immune responses to human infants [9]. Moreover, lambs develop similar lesions to those of human infants after RSV infection. Another advantage is that the neonatal period of lambs is longer than that in rodents providing a better opportunity to evaluate vaccine efficacy in the context of a newborn immune system. In the present study, we evaluated the immunogenicity of the ΔF/TriAdj vaccine formulation *via* two different routes of delivery in lambs. Additionally, we examined whether the ΔF/TriAdj vaccine is susceptible to interference by maternal antibodies (MatAbs), as well as the role of a booster dose, as a means of trying to overcome potential interference. The efficacy of the ΔF/TriAdj was equivalent when administered parenterally and mucosally, as well as in newborn lambs with and without MatAbs, demonstrating the potential of this vaccine in neonates.

2. Materials and methods

2.1. Vaccine formulation

The ΔF protein was produced and purified as described previously [6]. The ΔF protein was co-formulated with PCEP (250 μg), IDR1002 (500 μg) and polyI:C (250 μg) in PBS. PolyI:C (Invivogen, San Diego, CA) and IDR 1002 (Genscript, Piscataway, NJ) were formulated in a 1:2 ratio at 37 °C; poly[di(sodium carboxylatoethylphenoxy)]-phosphazene (PCEP) (Idaho National Laboratory, Idaho Falls, ID) along with ΔF protein was added after 30 min to make a final 1:2:1 ratio of polyI:C, IDR and PCEP (ΔF/TriAdj).

2.2. Animals and immunizations

Suffolk lambs were purchased from the Department of Animal and Poultry Science, University of Saskatchewan, SK, Canada. The trials were carried out according to the guidelines provided by the Canadian Council for Animal Care.

In the first trial, 42 lambs (two to three weeks old) were randomly allocated into six groups of seven animals each. Lambs were immunized twice intramuscularly (IM) at a three-week interval, with vaccine containing different amounts of ΔF (12.5, 25, 50 or 100 μg) formulated with TriAdj (250 μg polyI:C, 500 μg IDR and 250 μg PCEP). Two additional groups of lambs received either ΔF (100 μg) (Th2 control) or PBS (control). In the second trial, 13 lambs (two to three weeks old) were randomly allocated to three groups of five animals, and one group of three animals (control). Lambs were immunized twice IM or intrapulmonary (IP) at a three-week interval with vaccine containing ΔF (50 μg) formulated with TriAdj. The control group of lambs received PBS. In the third trial, 24 pregnant ewes were randomly allocated to two groups of 12 animals each, half of which were vaccinated twice IM with a three-week interval with ΔF/TriAdj, while the other half received TriAdj only. The lambs born to RSV F-immune or non-immune ewes were then given three IM vaccinations with either TriAdj or ΔF/TriAdj at three days, four weeks and eight weeks post-birth. Serum, colostrum and whole blood were collected at regular intervals, and bronchoalveolar lavages (BAL) at the end of the trial, to assess the humoral and cell-mediated immune responses.

2.3. Enzyme-linked immunosorbent assays (ELISAs)

RSV ΔF-specific IgG and IgA were evaluated in serum, colostrum and BAL by standard ELISA. Briefly, four-fold serially diluted samples were applied to ΔF-coated plates (50 ng/ml) and bound ΔF-specific antibodies were detected using alkaline phosphatase (AP)-conjugated goat anti-sheep IgG (Kirkegaard & Perry Laboratories, Gaitherburg, MD), and biotin-labeled IgA followed by streptavidin-AP (Jackson Immuno Research Laboratories Inc., Westgrove, PA). The assay was developed with *p*-nitrophenyl phosphate (Sigma-Aldrich Inc., St Louis, MO) substrate.

RSV ΔF-specific IgG avidity was determined by incubating ΔF protein-coated ELISA plates with serum, followed by washing with PBS or different concentrations of urea (7 M, 8 M and 9 M). The subsequent steps were performed in accordance with the standard ELISA protocol described above. The percentage of bound antibodies was calculated by comparing titers of bound antibodies after urea washes with titers after PBS washes.

A competitive ELISA was performed with biotinylated Palivizumab monoclonal antibody. Briefly, two-fold serially diluted sera (starting with 1:10 dilution) together with 100 ng/ml of biotinylated Palivizumab were applied to ΔF-coated plates and incubated for 2 h at room temperature. Bound Palivizumab was detected using streptavidin-AP and developed with *p*-nitrophenyl phosphate substrate. Optical densities (OD) of the wells were determined at 405–490 nm.

2.4. Virus neutralization (VN) assay

RSV-specific neutralization titers were determined by plaque reduction assays. Heat-inactivated sera were serially diluted in 96-well plates (Corning Incorporated, Corning, NY), and then mixed with 500 PFU/well of RSV strain A2 for 1 h at 37 °C. The serum-virus mixtures were transferred to duplicate HEp-2 cell monolayers and incubated at 37 °C, and after four days the cells were fixed and stained with 0.5% crystal violet.

2.5. Enzyme-linked immunospot (ELISPOT) assay

Isolation of peripheral blood mononuclear cells (PBMCs) and lymph node (LN) cells, and ELISPOT assays were performed by standard technique. Briefly, ELISPOT plates (Millipore, Billerica, MA) were coated overnight with a bovine gamma interferon (IFN-γ)-specific monoclonal antibody. PBMCs and LN cells (10^6 cells/well) were stimulated with either ΔF protein (2 μg/ml) or medium. The plates were incubated at 37 °C, and after 24 h bovine IFN-γ-specific rabbit serum [10] was added. Spots were developed using AP-conjugated goat anti-rabbit IgG (Kirkegaard & Perry Laboratories) and BCIP/NBT (Sigma-Aldrich) as the substrate.

2.6. Statistical analysis

All data were analyzed using GraphPad PRISM version 6 for Windows (GraphPad Software). Differences among all groups were examined using one-way ANOVA, followed by a Newman-Keuls post-test. Differences were considered significant if $P < 0.05$.

3. Results

3.1. Effect of the dose of RSV ΔF protein on the magnitude of the immune response

The goal of the first trial was to determine the optimal dose of ΔF protein in lambs. The lambs were immunized IM with 12.5, 25, 50 or 100 μg of ΔF protein formulated with TriAdj. Control lambs received 100 μg of ΔF protein in PBS or PBS alone. To determine the humoral immune responses, the RSV ΔF-specific serum IgG titers were determined. The lambs immunized with ΔF(50 μg)/TriAdj produced significantly higher ΔF-specific serum IgG titers compared to the other vaccinated groups, after both the first and the second immunization (Fig. 1A and B). The ΔF-induced secretion of IFN-γ by *in vitro* restimulated PBMCs was measured two weeks after the second vaccination. The ΔF(50 μg)/TriAdj formulation generated a significantly higher frequency of IFN-γ secreting cells