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Protection against respiratory syncytial virus by inactivated influenza virus carrying a fusion protein neutralizing epitope in a chimeric hemagglutinin

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Abstract

A desirable vaccine against respiratory syncytial virus (RSV) should induce neutralizing antibodies without eliciting abnormal T cell responses to avoid vaccine-enhanced pathology. In an approach to deliver RSV neutralizing epitopes without RSV-specific T cell antigens, we genetically engineered chimeric influenza virus expressing RSV $F_{262\text{-}276}$ neutralizing epitopes in the globular head domain as a chimeric hemagglutinin (HA) protein. Immunization of mice with formalin-inactivated recombinant chimeric influenza/RSV $F_{262\text{-}276}$ was able to induce RSV protective neutralizing antibodies and lower lung viral loads after challenge. Formalin-inactivated RSV immune mice showed high levels of pulmonary inflammatory cytokines, macrophages, IL-4-producing T cells, and extensive histopathology. However, RSV-specific T cell responses and enhancement of pulmonary histopathology were not observed after RSV infection of inactivated chimeric influenza/RSV $F_{262\text{-}276}$. This study provides evidence that an inactivated vaccine platform of chimeric influenza/RSV virus can be developed into a safe RSV vaccine candidate without priming RSV-specific T cells and immunopathology.

Key words: Influenza virus; Respiratory syncytial virus; Recombinant; Viral vector; F protein; Neutralizing epitope vaccine

Background

Respiratory syncytial virus (RSV) is a major cause of respiratory tract illness in infants and young children and responsible for hundred thousands of annual deaths globally.[1,2] RSV infection in early childhood has been linked to the recurrent wheezing later in life.[3] Moreover, immunocompromised patients and the elderly are also at significant risk for severe RSV disease. These significant morbidity and mortality associated with RSV underscore the urgent need for the development of an RSV vaccine.

In the 1960s, clinical trials in infants using formalin-inactivated RSV (FI-RSV) formulated with alum resulted in severe vaccine-enhanced pulmonary disease severity upon subsequent RSV infection.[4] Over the subsequent half century, previous studies about the pathogenesis of FI-RSV and the immune correlates of protection against RSV offer clues for development of a safe and effective RSV vaccine. A successful vaccine candidate will need to induce neutralizing antibodies, exclude immunosuppressive RSV proteins, and avoid the induction of undesirable T cell immune responses, which are known to be associated with vaccine-enhanced disease.[5–7] Despite the extensive effort, there are no licensed RSV vaccines or therapeutic agents other than palivizumab (Synagis®; MedImmune).

Palivizumab is a humanized monoclonal antibody specific for the antigenic site II of RSV fusion (F) protein and is the only

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product approved for the prevention of severe RSV disease in high-risk pediatric patients.[8] Passive transfer of monoclonal antibodies such as palivizumab and motavizumab suppresses viral replication *in vivo* and protects against RSV challenge in cotton rats without enhanced respiratory disease.[9,10] It has been reported that palivizumab prophylaxis is effective in reducing the frequency of hospitalizations due to RSV infection in children who are at high risk of acquiring severe RSV infection.[11] Nevertheless, due to the high cost of antibody prophylaxis, guidelines restrict recommendations for its use to the highest risk subgroups of infants.

Inactivated influenza vaccines have been safely used in humans. The reverse genetics system has provided a valuable tool for researchers to generate genetically manipulated influenza viruses expressing foreign antigens from different pathogens.[12] Previously, we and others have reported that foreign epitopes could be presented to the immune system in the context of a chimeric influenza virus protein using a live vaccine platform.[13–17] Here, we explored recombinant influenza virus as a safe inactivated vaccine for inducing neutralizing antibodies specific for the RSV F protein and avoiding RSV vaccine-induced abnormal immune responses. We generated recombinant influenza viruses carrying the RSV $F_{262\text{-}276}$ neutralizing epitope within the globular head domain of hemagglutinin (HA) protein. Recombinant chimeric influenza/RSV F in an inactivated vaccine platform was investigated regarding the immunogenicity, protective efficacy, cytokine and T cell responses as well as histopathology in comparison with FI-RSV.

Methods

Cells and viruses

HEp-2 cells and 293T cells were obtained from ATCC and propagated in Dulbecco's modified eagle media (DMEM) with 10% fetal bovine serum. Influenza A virus A/PR/8/1934 (H1N1, abbreviated PR8) virus was propagated in 10-day-old embryonated eggs. The RSV strain A2 was originally provided from Dr. Barney Graham. Purified inactivated viruses were produced by treating the virus with formalin at a final concentration of 1:4000 (v/v) as previously described.[15]

Construction of chimeric recombinant PR8/RSV HA-F

Plasmid pHW194-HA was previously described.[18] By introducing silent mutations, a *Pst*I restriction enzyme site was generated at nucleotide position 489 and removed at nucleotide position 74 of the PR8 HA gene. Moreover, new *Hind*III restriction enzyme site was introduced at nucleotide position 563 of the HA gene. The resulting plasmid was designated PR8-mHA. All silent mutations in the PR8-mHA were introduced by site-directed mutagenesis using the QuikChange Muli Site-Directed Mutagenesis Kit (Agilent Technologies, Böblingen, Germany) by use of a strategy similar to that employed by Li et al.[16,17] Three recombinant plasmids of chimeric HA-F constructs were generated by inserting the RSV $F_{772\text{-}825}$, $F_{784\text{-}828}$, and $F_{784\text{-}819}$ nucleotide fragment (Genbank accession number FJ614814) into the PR8-mHA plasmid using *Pst*I and *Hind*III restriction enzymes, respectively (Figure 1, *A*).

Recombinant viruses PR8/RSV HA-$F_{258\text{-}275}$, PR8/RSV HA-$F_{262\text{-}276}$, and PR8/RSV HA-$F_{262\text{-}273}$ were generated by reverse genetics using the pHW2000-based eight-plasmid system (kindly provided by R.G. Webster) as described by Hoffmann et al.[12] Briefly, 293T cells were cotransfected with eight pHW2000 plasmids containing eight influenza virus gene segments including the chimeric HA-F constructs (Figure 1, *A*). After 48 h transfection, the supernatant was collected and further inoculated into embryonated chicken eggs. Seventy-two hours after inoculation, the presence of the recovered recombinant viruses was proved by hemagglutination of chicken red blood cells. To determine the incorporation of the antigenic site II of the RSV F protein into recombinant HA-F chimeric proteins, the reactivity to palivizumab (MedImmune, Gaithersburg, MD) was analyzed by enzyme-linked immunosorbent assay (ELISA).

Immunizations and RSV challenge of mice

For animal experiments, six- to eight-week-old female BALB/c mice ($n = 5$; Charles River Laboratories) were immunized intramuscularly with 10 μg of formalin-inactivated PR8/RSV HA-$F_{262\text{-}276}$ virus or 2 μg of inactivated PR8/RSV HA-$F_{262\text{-}276}$ virus alone or mixed with 50 μg of aluminum hydroxide (alum) adjuvant or 2 μg of inactivated PR8 wild-type (PR8 WT) virus. The FI-RSV control group was intramuscularly immunized with 2 μg of FI-RSV in alum adjuvant.[19] Blood samples were obtained three weeks after each immunization. Immunized mice were challenged with RSV A2 strain (2×10^5 PFU) at 4 weeks after boost immunization. All animal experiments presented in this study were approved by the Georgia State University IACUC review boards (IACUC A14025).

Assays for antibody responses and virus titration

RSV F protein-specific antibodies (IgG, IgG1, and IgG2a) were determined in samples by enzyme-linked immunosorbent assay (ELISA) as previously described.[20] To determine hemagglutination inhibition (HI) titers, serum samples were incubated with receptor destroying enzyme (RDE, Denka Seiken, Japan) and heated at 56 °C. In brief, HI titers were determined using inactivated PR8 virus and 1% chicken erythrocyte suspension with two-fold diluted serum samples after RDE treatment.

RSV-specific neutralizing antibody titers in immune sera were evaluated by a standard method as previously described.[21] Briefly, the serum samples were heat-inactivated at 56 °C and serially diluted two-fold in serum-free DMEM. Equal volumes of RSV (300 PFU/well) were mixed with diluted sera. A mixture of RSV with or without immune sera was incubated at 33 °C, 5% CO_2 for 1 h prior to incubation in the HEp-2 cell monolayers. The next steps were followed by an immune-plaque assay procedure as described previously.[15] After fixing with 5% formaldehyde in PBS and blocking with 5% non-fat dry milk in PBST, anti-RSV F monoclonal antibody (131-2A, Millipore) and then HRP conjugated anti-mouse IgG antibody were used. Individual plaques were developed using 3,3'-diaminobenzidine tetrahydrochloride (DAB) substrate (Invitrogen, Camarillo, CA) and then counted.