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Alphavirus replicon particles encoding the fusion or attachment glycoproteins of respiratory syncytial virus elicit protective immune responses in BALB/c mice and functional serum antibodies in rhesus macaques

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Abstract

Respiratory syncytial virus (RSV) is a major cause of acute respiratory tract disease in humans. Towards development of a prophylactic vaccine, we genetically engineered Venezuelan equine encephalitis virus (VEEV) replicons encoding the fusion (Fa) or attachment (Ga or Gb) proteins of the A or B subgroups of RSV. Intramuscular immunization with a formulation composed of equal amounts of each replicon particle (3vRSV replicon vaccine) generated serum neutralizing antibodies against A and B strains of RSV in BALB/c mice and rhesus macaques. When contrasted with purified natural protein or formalin-inactivated RSV formulated with alum, the 3vRSV replicon vaccine induced balanced Th1/Th2 T cell responses in mice. This was evident in the increased number of RSV-specific IFN-γ^+ splenocytes following F or G peptide stimulation, diminished quantity of eosinophils and type 2 T cell cytokines in the lungs after challenge, and increased *in vivo* lysis of RSV peptide-loaded target cells. The immune responses in mice were also protective against intranasal challenge with RSV. Thus, the replicon-based platform represents a promising new strategy for vaccines against RSV.

Keywords: Respiratory syncytial virus; Vaccine; Venezuelan equine encephalitis virus; Alphavirus; Replicons; BALB/c mice; Rhesus macaques; Balanced immune responses; Bronchoalveolar lavage

1. Introduction

Respiratory syncytial virus (RSV) is a single-strand, negative sense RNA virus of the family Paramyxoviridae, subfamily Pneumoviridae [1,2], and a major cause of seasonal epidemics of severe lower respiratory tract disease in humans [3]. Worldwide an estimated 64 million RSV infections occur each year that result in 160,000 deaths [4]. While RSV attacks all age groups, the most severe disease occurs in very young infants, aged adults, and those at risk because of immunosuppression or chronic cardiopulmonary diseases. In the US, approximately 125,000 infants are hospitalized annually with acute lower respiratory tract disease caused by RSV, leading to nearly 500 deaths [5]. The estimates of annual RSV-related hospitalizations (177,525) and deaths (14,000) in adults and other at-risk subjects approach that of influenza [6,7]. Despite 50 years of

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research, a vaccine for RSV is still not available for human use [8,9].

The inability to successfully develop a vaccine results from several factors. The exacerbated disease associated with a formalin-inactivated RSV (FI-RSV) vaccine [10,11] significantly curtailed subunit vaccine development for RSV-naïve infants. This exacerbated disease is thought to have occurred because subunit vaccines, at least those formulated with alum adjuvant, primarily induce type 2 helper T cell responses in animals. The generation of balanced immune responses characterized by equivalent type 1 (Th1) and type 2 (Th2) T cell responses and elicitation of both humoral and cellular immunity is a central caveat of an ideal RSV vaccine. Unbalanced Th2-biased T cell responses following immunization of RSV-naïve infants with the FI-RSV vaccine was believed to play a role in exacerbated respiratory tract disease [12,13]. Subunit vaccines may be, however, appropriate for seropositive infants, children and adults that are at increased risk for disease. Indeed, several candidate vaccines were advanced to Phase II clinical studies [8,14]. However, these efforts were discontinued because of poor immunogenicity or issues with safety. Several strategies were also used to develop attenuated RSV vaccines for naïve infants. These included manipulation of the RSV genome by serial passage at cold temperature and chemical mutagenesis [5,9], reverse genetics [9,15–17], or by gene deletion [18–21]. Unfortunately, a live attenuated vaccine acceptable for use in infants during the first few weeks of life has thus far not been realized [8,14,22]. Experimental vaccines based on DNA or viral vectors genetically manipulated to express RSV genes [5,22–24] were also explored without success.

Given the disappointing results from previous approaches to RSV vaccine development, we explored the potential of "replicon" technology based upon an attenuated strain of Venezuelan equine encephalitis virus (VEEV). VEEV is an alphavirus, and as such contains a positive sense, single-strand RNA genome containing two ORFs that respectively encode the non-structural proteins (nsP1–4) required for replication and transcription, and the capsid and envelope structural proteins important in viral assembly and attachment [25]. A major advantage of the VEEV replicon technology is the strong 26S subgenomic promoter that controls transcription of the structural genes. Replacement of VEEV structural genes with foreign genes enables an increased production of vaccine antigens *in situ*. Safety is a second advantage of the technology and is achieved through the use of a bipartite helper RNA system to produce VEEV replicon particles [26]. In this system, the structural genes are provided *in trans* in the packaging cells in the form of two helper RNAs encoding either capsid or envelope glycoproteins.

Our strategy focused on replacement of the VEEV structural genes with those of the major antigenic determinants of RSV, the F and G envelope glycoproteins [27–30]. F protein is a 574 amino acid type I membrane glycoprotein that is highly conserved between isolates from A and B subgroups and mediates fusion with the membrane of bronchial epithelial cells [31–35]. In contrast, the RSV G protein is a type II membrane glycoprotein composed of 289–299 amino acids. G protein is produced in soluble and membrane bound forms [35–37] and believed to facilitate attachment of the virus particle to the epithelial cell [38]. The significant amino acid diversity in G protein amongst RSV isolates determines A and B subgroup specificity [39]. Herein, we report on the genetic construction and formulation of a multi-component VEEV replicon-based vaccine for RSV. Because strains representing both A and B subgroups of RSV [40,41] often co-circulate during the winter months, and both are associated with varying degrees of disease severity, we constructed three VEEV replicon particles encoding either F protein (Fa) or G protein (Ga) from an A subgroup, or G protein (Gb) from a B subgroup. We demonstrate that the resultant particles co-formulated as a multi-component vaccine (3vRSV) are immunogenic in BALB/c mice and rhesus macaques. When contrasted with subunit vaccines prepared with purified natural protein or FI-RSV and alum adjuvant, the 3vRSV replicon vaccine elicited more balanced T cell responses in BALB/c mice. Thus, the replicon-based platform represents a promising new strategy for immunization against RSV.

2. Materials and methods

2.1. Cells and viruses

HEp-2 cells (American Type Culture Collections [ATCC, CCL-23], Rockville, MD), and Vero cells (ATCC, CCL-81) were cultured (37 °C, 5% CO_2) in Dulbecco's minimal essential medium (D-MEM) supplemented with 5% FBS, 2 mM L-glutamine, and 1X penicillin–streptomycin (Invitrogen, Carlsbad, CA). The A2 [42], and V95-11 (B subgroup kindly provided by Dr. Peter Wright, Vanderbilt University) strains of RSV were used in the studies. A recombinant attenuated vesicular stomatitis virus (VSV) expressing HIV Gag protein (rVSViN3CT1-Gag5) was kindly provided by Dr. David Clarke, Wyeth Research.

2.2. RSV replicon construction

ORFs encoding the F or G glycoproteins were generated by PCR using template plasmid obtained by reverse transcribing segments of RSV strains A2 and V95-11 genomes and cloning the resulting cDNAs into a Bluescript vector. The following primers were used in the amplification reactions: RSV Ga Kozak forward (5′ ATTGGGGATATC**GCCACC**A<u>TG</u>TCCAAAAACAAGGACCAACG 3′), RSV Ga Kozak reverse (5′ ATGTTTTGGCGCGCCTACTGGCGTGGTGTGTTGGGTGGAGAT 3′), RSV Fa Kozak forward (5′ AACTCTGATATC**GCCACC**<u>ATG</u>GAGTTGCTAATCCTCAAAGC 3′), RSV Fa reverse (5′ AGGTGCTGGCGCGCCTTAGTTACTAAATGCAATATTATTTATAC