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Respiratory Syncytial Virus Vaccine Development

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Abstract

Respiratory syncytial virus (RSV) is a clinically significant cause of respiratory tract disease, especially among high-risk infants and immunocompromised and elderly adults. Despite the burden of disease, there is no licensed prophylactic RSV vaccine. The initial efforts to develop an RSV vaccine involved formalin-inactivated virus preparations that unexpectedly caused vaccine-enhanced disease in clinical trials in RSV-naïve children. Over the last four decades, cautious and deliberate progress has been made towards RSV vaccine development using a variety of experimental approaches (Table 1), including live attenuated strains, vector-based, and viral protein subunit/DNA-based candidates. The scientific rationale, preclinical testing, and clinical development of each of these approaches are reviewed.

Keywords

respiratory syncytial virus; vaccine; humoral immunity; cellular immunity; respiratory tract infection

Introduction

Since its identification in 1956, RSV has been known to be a significant cause of respiratory tract illness in persons of all ages and is the most clinically important cause of lower respiratory tract infections in infants and children¹. Following primary RSV infection, which generally occurs by age 2, immunity to RSV remains incomplete and frequent re-infections occur throughout life, with the most severe infections occurring at the extremes of age and among the immunocompromised². In the United States, RSV is estimated to cause approximately 126,000 annual hospitalizations and approximately 300 deaths among infants younger than 1 yr of age³. Furthermore, RSV accounts for more than 80,000 hospitalizations and more than 13,000 deaths each winter among adults who are elderly or have underlying cardiopulmonary and/or immunosuppressive conditions⁴. Despite such burden of disease, the number of currently licensed prophylactic and therapeutic agents against RSV infection remains exceedingly limited -- the humanized monoclonal antibody (mAb) palivizumab, which is currently licensed only for use in high-risk infants, and ribavirin that is licensed for use only in the pediatric population¹. Because of marked imbalance between the clinical burden of RSV

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and the available therapeutic and prophylactic options, development of an RSV vaccine remains an unmet medical need.

RSV is an enveloped virus of the *Paramyxoviridae* family⁵. Clinical RSV isolates are classified according to antigenic group (A or B) and further subdivided into 5–6 genotypes based on the genetic variability within the viral genome. Each virion contains a non-segmented, negative sense, single-stranded RNA that encodes 11 proteins, eight being structural and three are non-structural (NS1, NS2, M2-2). The viral envelope bears three transmembrane glycoproteins (G, F, SH) as well as the matrix (M) protein. Within the envelope, viral RNA is encapsidated by a transcriptase complex comprised of the N (nucleocapsid), P (phosphoprotein), M2-1 (transcription elongation factor), and L (polymerase) proteins. The early events in RSV replication are: 1) viral attachment to the target cell, a process mediated mainly by the attachment (G) glycoprotein, and 2) membrane fusion and viral penetration into the host cell, processes that require the fusion (F) protein and augmented by the SH protein. Among viral isolates, some RSV-encoded proteins such as F are highly conserved with respect to amino acid (aa) sequence while others such as G display extensive antigenic variation between and within the two major antigenic groups.

The various RSV-encoded proteins have been extensively analyzed with respect to their immunogenicity. Several proteins, including N, M2-1, NS1, and F, bear epitopes that induce cytotoxic T lymphocyte (CTL) responses in murine- and/or human-derived lymphocytes⁵. In contrast, extensive efforts to identify CTL epitopes in other proteins, including the G protein, have been unfruitful. With regard to humoral response, only antibodies against F or G are neutralizing and confer resistance to RSV upon passive transfer in animal models^{6, 7}. A number of F-specific neutralizing monoclonal antibodies (mAbs) also possess the ability to inhibit viral fusion activity⁸. One such mAb is palivizumab, a humanized derivative of an anti-F neutralizing mAb that is licensed for prevention of serious RSV illness in high-risk children⁹.

RSV immune responses; balance between protection and disease pathogenesis

In the infected host, RSV stimulates a broad range of innate and adaptive immune responses, including chemokine and cytokine secretion, neutralizing humoral and mucosal antibodies, and type 1 and type 2 CD4+ and CD8+ T cells^{5, 10}. These host immune responses, in turn, are thought to be primarily responsible for the clinical manifestations of RSV infections since RSV causes limited cell cytopathology *in vivo*¹⁰. Based on extensive testing in animal models and also from human immunological studies, the phenotypic manifestations and severity of RSV disease are mediated by the fine balance and dynamic interactions among the various chemokine, cytokine, and cellular responses¹¹.

Within the host, cellular and humoral responses appear to play different roles in the protection against and resolution of RSV infection as well as disease pathogenesis. As shown by clinical and preclinical studies with palivizumab, RSV-specific antibodies are sufficient to prevent or limit the severity of infection but not required for clearing primary infection⁹. However, once RSV infection is established, T cell-mediated responses are necessary to abolish viral replication¹². In murine models, passive transfer of CD4+ T-cells and CD8+ CTL eliminates RSV, the latter being more efficacious¹³. Additionally, T cell responses play key roles in pulmonary pathology during infection, with striking differences noted between Th1 and Th2 CD4+ cells. Interferon- γ (IFN- γ) secreting CD4+ T cells (Th1), with or without associated CD8+ CTL response, clear virus with minimal lung pathology, while viral clearance by IL-4 secreting CD4+ cells (Th2) is associated with more significant pulmonary changes, often marked by eosinophilic infiltration^{14, 15}.

Of profound relevance to vaccine design is the effect of the priming immune response on clinical illness and lung pathology during subsequent RSV infection. Immunization with live RSV or with replicating vectors encoding RSV F protein induces a Th1 dominant response with neutralizing antibody and CD8+ CTL responses that are associated with minimal pulmonary pathology upon virus challenge^{11, 16}. In contrast, immunization with inactivated RSV preparation induces a Th2 dominant response without associated CD8+ CTL but paradoxically leads to increased pathological changes in the lungs¹⁷. Interestingly, the administration of RSV G protein as a purified subunit vaccine or even in the context of a replicating vector induces a Th2-dominant response and leads to eosinophilic pulmonary infiltrates and airway hyper-reactivity following virus challenge¹⁸. It should be noted that these observations are strikingly reminiscent of the first RSV vaccine studies using formalin-inactivated virus (see below).

Factors to be considered for RSV vaccine development

Extensive review of RSV replication, pathogenesis, and immune response in animal models and human infections has identified several key issues for RSV vaccine development¹⁹. First, the immune system of young infants, the primary population for vaccination, is notable for its immaturity and the presence of maternal anti-RSV antibodies during the first several months of life; in turn, these two factors may negatively affect the emergence of a robust immune response following vaccination. Second, a successful RSV vaccine must be able to target both A and B subtypes; this requirement is hampered by the genetic variability of RSV genome, and particularly of the RSV G protein, as well as the post-translational glycosylation of F and G proteins. Third, immunity against RSV remains incomplete after natural infection and thus annual vaccinations may be required; the goal of such vaccination programs is to prevent severe lower respiratory tract infections. It is possible that two vaccines – one for the RSV-naïve, infant population and another for RSV-experienced adults – may be required. Lastly, in the historical context of formalin-inactivated RSV vaccine trials (see below), the safety profile of RSV vaccine candidates will need to be well established during preclinical and clinical development.

Prior and current RSV vaccine approaches

Various strategies have been pursued to develop an effective and safe RSV vaccine including: 1) inactivated virus preparations; 2) live attenuated/genetically engineered viruses; 3) purified RSV protein subunit vaccine preparations; 4) vector-based vaccine candidates; and 4) DNA-based vaccines. Each approach is summarized below.

Inactivated virus preparations

The first vaccine trial, performed nearly 40 years ago, employed a formalin-inactivated whole virus preparation (FI-RSV) with aluminum hydroxide adjuvant²⁰. Strikingly, FI-RSV failed to induce neutralizing antibodies or protect vaccinated infants and unexpectedly led to enhanced disease severity when RSV infection occurred during the subsequent winter; in one center, more than 80% of the recipients of the FI-RSV vaccine required hospitalization as compared to only 5% of RSV-infected control subjects^{21–23}. Such disease enhancement, or potentiation, in which the vaccination paradoxically worsened the clinical course of subsequent RSV infection, appears to be due to altered host immune response against RSV.

The lack of protective efficacy of FI-RSV is likely due to one or more factors, including the development of poorly neutralizing antibodies against RSV-encoded epitopes, perhaps due to denaturation of such epitopes; incomplete affinity maturation of anti-RSV antibodies; and lack of a robust anti-RSV CTL response as shown in animal studies^{24–26}. The potentiation phenomenon appears to be due to a vaccine-induced priming and delayed hypersensitivity

response involving Th2 CD4+ T-cells²⁶. It should be noted that a clinical trial using parenterally administered live virus also failed to provide protection, although enhanced RSV disease did not occur²⁷.

Live attenuated/genetically engineered RSV derivatives

The major goal of generating live-attenuated RSV strains is to create a vaccine with the capacity to elicit a broad, protective immune response without significant clinical illness. Such studies of live-attenuated RSV strains began with the isolation of RSV strains that are able to replicate better at < 37°C (i.e. at temperatures similar to those within the upper respiratory tract) than at 37°C (i.e. within the lower respiratory tract), thereby reducing the risk of serious lower respiratory tract illness. To date, such strategies include: 1) serial viral passages to identify and characterize cold-passaged (*cp*) strains; 2) chemical mutagenesis to generate mutant RSV strains with temperature-sensitive (*ts*) phenotypes; and 3) reverse genetics to engineer recombinant RSV strains bearing attenuated phenotype while maintaining genetic stability²⁸.

Extensive serial passage at progressively lower temperatures led to the isolation of *cp*RSV, which remained replication-competent at 26°C²⁹. Derivatives of *cp*RSV strains were generated by mutagenesis with 5-fluorouracil and one such derivative, *cpts248/404*, was deemed sufficiently attenuated for clinical testing in RSV-naïve infants (1–2 months old)³⁰. In such a study, >80% of infant subjects were infected by the attenuated RSV strain and had a ≥ 4-fold increase in RSV-specific IgA levels following challenge. Moreover, the majority of vaccine recipients were resistant to infection by a second dose of the vaccine strain given one month after the primary challenge. However, > 70% of 1–2 month old infant vaccine recipients developed nasal congestion at the time of peak viral titer. Thus, the phenotype of *cpts248/404* strain was deemed as insufficiently attenuated for use in very young, RSV-naïve infants. However, the replication of this viral strain appeared to be unrelated to the level of maternal antibodies among vaccinees and there was no obvious evidence of vaccine-enhanced RSV disease in post-study follow-up.

Another group of biologically derived RSV mutants was isolated from successive rounds of chemical mutagenesis of the group A strain RSS-2 and selection for *ts* phenotype. The final derivative, *ts1C*, was tested in intranasal challenge in a small study involving 22 healthy young adults³¹. Following such administration, 30% of vaccine recipients exhibited viral shedding and among this subgroup, none demonstrated clinical signs and symptoms of respiratory diseases. Following intranasal challenge, nearly 70% exhibited a ≥ 2-fold increase in serum titers of RSV-neutralizing antibodies, with the greater increases trending to correlate with lower pre-vaccination anti-RSV titers. Thus, this small-scale study provided support for increased study of this RSV strain in the adult population.

The advent of reverse genetics and elucidation of structure-function relationships for the majority of RSV-encoded proteins have led to the generation and testing of second-generation live-attenuated vaccine candidates. To this end, the genomic RNA of live-attenuated RSV strains that were biologically derived and/or chemically mutagenized were sequenced to identify nucleotide changes potentially associated with the attenuated phenotype^{32–34}. Such nucleotide changes, e.g. mutation affecting aa 1030 of the L protein, have then been engineered into existing RSV strains in hopes of further attenuation. Deletions of RSV genes have also been attempted for further attenuation and/or potentially increasing host response; for example, the deletion of the SH gene causes up to 10 fold decrease in RSV replication in primates, while the deletion of NS1 and/or NS2 genes may increase the expression of type I interferon and thus potentially increase immunogenicity in vaccinees^{35, 36}.

Based on the above genetic manipulations, at least two lineages of second-generation, live-attenuated RSV vaccine candidates have been generated. The first is rA2*cp*248/404/1030ΔSH,

in which the cp248/404 was engineered to bear the L protein aa 1030 mutation as well as the deletion of the SH gene³². When evaluated in RSV-naïve, very young infants (1–2 months old), ≥ 4 fold increase in anti-RSV antibodies were noted in 44% of infants previously uninfected with RSV, and the second dose of the vaccine strain given within two months of the priming dose was significantly restricted, indicating that protective immunity was achieved in a majority of RSV-naïve vaccine recipients³⁷. However, the rA2cp248/404/1030 Δ SH strain appears to exhibit some degree of genetic and phenotypic instability during replication, likely necessitating further manipulations for additional vaccine testing³⁷. The second line of reverse genetics-mediated RSV vaccine candidate is comprised of rA2cp derivative bearing deletions in the NS2 gene, i.e. rA2cp Δ NS2, rA2cp530/1009 Δ NS2, and rA2cp248/404 Δ NS2³⁸. In limited clinical studies, the first of the three strains appeared to be overly attenuated in adults, while the latter two were over-attenuated for use in the pediatric population³⁸. Thus, derivatives of RSV strains deleted for NS2 are being studied for additional genetic manipulations designed to achieve a compromise between over- and under-attenuated phenotype.

Taken together, the live-attenuated RSV vaccine approach has shown promise based on current knowledge of *cp/ts* mutations within the genome, essential/non-essential RSV genes, and structure/function of RSV-encoded genes. The major challenge to this approach remains the need of achieving an appropriate balance between the two priorities of live-attenuated RSV vaccine development (over-attenuation/suboptimal immunogenicity and under-attenuation/bearing pathogenic potential)¹⁹.

Subunit vaccines

RSV-derived F and G proteins and their derivatives bearing neutralizing epitopes have undergone preclinical and clinical assessments as non-replicating protein subunit vaccines. The first of such preclinical studies utilized RSV F and G proteins that were purified from RSV-infected cell cultures and F and F/G chimeric proteins produced in baculovirus-infected insect cells or transfected mammalian cell lines^{39, 40}. Rodents immunized with these preparations generated antibodies similar to those observed for FI-RSV in that despite recognition of RSV antigen in ELISAs, the resulting antibodies were poorly neutralizing^{41, 42}. In addition, rodents immunized with such RSV-encoded proteins and subsequently challenged 3–6 months later developed lung pathology similar to those observed for FI-RSV⁴². To circumvent the potential deleterious consequences of subunit vaccination, several groups have utilized various adjuvants, including those specific for various Toll-like receptors (TLRs) (e.g. monophosphoryl lipid A (MPL) for TLR-4, CpG oligonucleotides for TLR-9) and have reported that in some cases, the use of adjuvants can significantly alter the immunogenicity and adverse effects of subunit vaccines^{43, 44}. Of relevance is the recent observation that TLR agonists may play a significant role in the affinity maturation of antibodies against RSV-encoded proteins⁴⁵. Thus, in principle, appropriate TLR stimulation during vaccination with RSV subunit preparations may generate high-affinity anti-RSV antibodies that recognize appropriate epitopes in non-denatured configurations.

With respect to clinical evaluation of RSV subunit vaccine candidates, several preparations of RSV-encoded proteins have been tested. Such preparations include: PFP (purified F protein) derivatives (PFP-1, -2, and -3); FG chimeric protein; a co-purified formulation of F, G, and M proteins, and a bacterially derived RSV G derivative (BBG2Na). Such efforts are briefly described below.

The PFP series have been purified from RSV-infected Vero cells and successive versions are comprised of higher purity of RSV F (i.e. 90–95% F for PFP-1 vs. > 98% for PFP-2 and -3)^{46, 47}. PFP-1 has been tested in RSV-seropositive children in two clinical trials involving 24–48 month old and 18–36 month old, RSV-seropositive children^{47, 48}. In these studies, no

obvious adverse events were evident and ≥ 4 -fold increase in RSV-neutralizing antibody titer was observed in a majority of subjects. A single priming intramuscular dose of PFP-2 (50 μ g) was administered to 1–12 year old children with bronchopulmonary dysplasia ($n = 10$) or 1–8 year olds with cystic fibrosis ($n = 17$); in this study, the majority of participants exhibited ≥ 4 fold increase in RSV-neutralizing antibody titers^{49, 50}. PFP-3 has been tested as a single 30 μ intramuscular vaccination dose in a phase II study among 143 1–12 year old children with cystic fibrosis⁵¹. As in the case of PFP-2, PFP-3 also elicited a ≥ 4 -fold increase in RSV neutralizing titers. However, the rate of RSV infection after vaccination was not significantly different between the vaccinated and control groups. In two studies involving elderly adults, a single 50 μ g dose of PFP-2 was also immunogenic in 61% (out of 33 subjects > 60 years old) and 47% (of 36 frail elderly adults > 65 years old)^{52, 53}. Lastly, a single 20 μ g dose of PFP-2 administered to 20 pregnant women was well tolerated⁵⁴. Although the vaccine led to a ≥ 4 -fold increase in anti-F antibodies in the vast majority of vaccinees and their infants up to 6 months following birth, only 10% of the study subjects had a corresponding ≥ 4 -fold increase in RSV neutralizing antibody titers.

BBG2Na, a bacterially derived protein bearing aa 130–230 of subgroup A RSV G protein fused to the albumin-binding domain of streptococcal protein G, has also undergone preclinical and clinical evaluation. In rodent models, BBG2Na conferred protection against RSV even though it generated modest levels of RSV-neutralizing antibodies⁵⁵. However, in limited primate studies, this antigen was associated with limited viral challenge protection and also with Th2-biased immune response⁵⁶. Among healthy young adults and elderly adults, this antigen was also well tolerated in phase II studies¹⁹. However, in a phase III study involving elderly adults, anti-RSV serum antibody titers declined significantly within four weeks after vaccination and limited number of unanticipated adverse events have been reported.

With respect to other clinical trials with RSV subunit/protein-derived vaccine candidates, limited data are available. For FG chimeric protein testing, phase I and II studies involving its administration with various adjuvants have been started but not formally reported¹⁹. In one study, a co-purified formulation of RSV F, G, and M proteins were emulsified in one of two adjuvants (aluminum hydroxide and poly(di[carboxylatophenoxy]phosphazene; PCPP) and administered to healthy adults (total $n = 70$)⁵⁷. In this study, both vaccine formulations induced similar levels of ≥ 4 -fold increase in RSV neutralizing antibody titers and collectively in $> 80\%$ of the study population. In a large-scale phase II study in which 1169 elderly adults with cardiopulmonary conditions, the RSV F, G and M-containing vaccine was immunogenic; a greater proportion of subjects immunized with non-adjuvanted vaccine had ≥ 4 -fold increase in RSV neutralizing antibody titers as compared to that in the group challenged with alum-containing vaccine candidate (168/383 (44%) vs. 129/400 (33%))⁵⁸.

Short peptides derived from RSV F and G proteins have been tested in animal models. In the case of short, RSV F-derived peptides, limited immunogenicity was seen⁵⁹. Recent data suggest that in mice, administration of bacterially derived short fragments of RSV F (including aa 255–278 and 412–524) fused to the ctxA(2)B cholera holotoxin led to anti-F, RSV-neutralizing antibody response and a modest protective efficacy in mouse RSV challenge studies^{60, 61}. For RSV G, the aa sequence (174–187) derived from the central core of RSV G has been administered to mice; in such experiments, protections against RSV infection was noted despite inefficient RSV neutralizing response^{62, 63}.

An alternative platform for RSV subunit vaccines is virus-like particles (VLPs), which are non-replicating, non-infectious particles derived from virus-encoded proteins⁶⁴. In the case of human papillomavirus (HPV) VLP-based licensed vaccine, the HPV L1 capsid protein self-assembles into particles that appear morphologically indistinguishable from native HPV virions⁶⁵. The resulting VLPs elicit strong humoral and cellular immune responses and provide

durable protection against HPV. Other virus-encoded proteins, such as the influenza hemagglutinin and the hepatitis B surface antigen (HBsAg), can also form VLPs and in the latter case, have been engineered to contain an RSV-encoded CTL epitope⁶⁶. Most recently, nanoparticles derived from bacterially derived RSV nucleocapsid proteins have been shown to confer a moderate degree of protection against RSV challenge in rodent models⁶⁷. Because of their potent immunogenicity, VLPs may be an alternative platform for RSV subunit vaccine provided that appropriate epitopes can be successfully presented.

Vector-based RSV vaccines

Several live virus vectors have been generated to express RSV-encoded proteins. Such vectors have included vaccinia virus (mouse-adapted WR strain and the MVA strain) engineered to express RSV F and G as well as adenovirus derivatives expressing RSV F^{68, 69}. In these cases, there was no significant immunogenicity or protective efficacy in primates^{70–72}. More recently, genetically engineered varieties of parainfluenza viruses including HPIV3, Sendai virus (SeV), and a chimeric bovine parainfluenza virus (BPIV)/ HPIV3 have been used to express RSV F and G under the control of endogenous PIV gene expression signals; these have the possibility of being developed as a bivalent RSV/PIV vaccine for the pediatric population^{73–76}. B/PIV3 expressing RSV F has been shown to be immunogenic and efficacious in RSV protective challenge studies in primates and is currently being evaluated in clinical studies⁷⁷.

Recent studies have described various strategies to express RSV F and/or G in the context of live bacterial vectors and non-replicating viral vectors. For example, *Staphylococcus carnosus*, a non-pathogenic bacterium, has been modified to express on its surface three portions of the RSV G protein fused to a fragment of the cholera B subunit protein⁷⁸. The resulting bacteria have the potential advantage of oral or intranasal administration but were found to provide limited and variable protective efficacy in mouse RSV challenge studies. RSV F and/or G have been expressed in the context on replication-incompetent (i.e. replicon-based) alphavirus vectors, as well as the avian Newcastle disease virus and human rhinovirus type 14^{79–82}. Most of these viral vectored RSV vaccine candidates are immunogenic and protective to varying degrees in mouse RSV challenge studies.

DNA vaccines

Through various routes, including intramuscular, intradermal, microparticle “gene gun,” or DNA-carbohydrate/albumin intranasal approaches, immunization of rodents with DNA plasmids encoding RSV F or G proteins has shown that administration of DNA is immunogenic and protective to a limited degree in RSV challenge experiments^{83–86}. DNA-based RSV vaccine has the potential to express RSV-encoded proteins in its native structure and possibly do so in the context of immature immune system in infants and thereby overcoming the maternal antibody-associated immunosuppression of anti-RSV response. However, lingering technical challenges for this approach include: the time of onset of immune response, the amount of DNA required to generate a clinically relevant immune response, and the likely need for multiple repeated administration of such DNA molecules.

Summary

RSV vaccine development remains a high priority based on the burden of diseases and limited number of licensed prophylactic and therapeutic options. Previous and current efforts involve the development and preclinical/clinical (where appropriate) testing of live attenuated RSV strains, RSV subunit-based proteins, live/non-replicating vector-derived vaccine candidates, and DNA-based approaches. Based on the historical experience with formalin-inactivated RSV vaccine, further studies on RSV vaccine development will likely require continued safety and

efficacy monitoring. Since immune response against RSV following natural infection is incomplete, it is possible that two vaccines – one for pediatric, RSV-naïve subjects and another for RSV-infected elderly adults – will be developed.

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Table 1

Overview of various experimental approaches to RSV vaccine development.

Experimental approach	Preclinical study comments	Clinical study comments
Non-replicating, inactivated virus		
Formalin-inactivated		Associated with potentiation of RSV disease among RSV-naïve infants and children; testing discontinued
Subunit-based		
Purified RSV F protein (PPF series)		Elicits anti-RSV antibodies in RSV-seropositive children and adults In general, an inverse correlation between homogeneity of RSV F protein preparation and subsequent immunogenicity
F/G/M co-formulation		Phase II testing: immunogenic in healthy and elderly high-risk adults
BBG2Na	Confers protection in rodent models despite modest levels of RSV-neutralizing antibodies	Phase II testing: immunogenic Phase III testing: discontinued
RSV F and G proteins purified from RSV-infected cells	In general: elicits poorly neutralizing antibodies and can lead to pulmonary pathology reminiscent of FI-RSV in animal models Use of TLR agonists may significantly alter immunogenicity and adverse effects in rodent models	
Virus-like particles	HBsAg derivatives bearing RSV M2 CTL epitope generated RSV nucleocapsid protein complexes elicit protective efficacy in mice	
F/G chimeric proteins		Phase I/II studies started but not reported
RSV F- and G-derive peptides	Short F-derived fragments fused to cholera holotoxin elicited RSV-neutralizing antibodies and modest protective efficacy in mice RSV G-derived peptides generated protective efficacy despite limited RSV neutralizing response in mice	
Vector-Based		
Vaccinia virus expressing RSV F and G	No significant immunogenicity or protective efficacy in primates	
Adenovirus expressing RSV F	No significant immunogenicity or protective efficacy in primates	
HPIV3, Sendai virus, BPIV/HPIV 3 expressing	B/PIV3 + RSV F immunogenic and efficacious in primates	Currently under clinical investigation

Experimental approach	Preclinical study comments	Clinical study comments
RSV F and G		
<i>Staphylococcus carnosus</i> expressing portions of RSV G	Limited and variable protective efficacy in mice	
RSV F and G expressed using replication-noncompetent alphavirus vectors, avian Newcastle disease virus, human rhinovirus 14	Immunogenic and protective to varying degrees in mice	
DNA-based		
DNA plasmids expressing RSV F or G	Various routes of administration Immunogenic and protective to varying degrees in animal models	
Live-attenuated and genetically engineered RSV derivatives		
Cold-passaged (<i>cp</i>) and temperature-sensitive (<i>ts</i>) phenotypes		Immunogenic in RSV-naïve and –seropositive subjects
Second-generation derivatives (bearing gene deletions and/or attenuating mutations) based on reverse genetics		Challenges of phenotype (over- or under-attenuation in clinical studies) and genotype (genetic instability/variability during host replication)