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Biological Challenges and Technological Opportunities for Respiratory Syncytial Virus Vaccine Development

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Summary

Respiratory syncytial virus (RSV) is an important cause of respiratory disease causing high rates of hospitalizations in infants, significant morbidity in children and adults, and excess mortality in the elderly. Major barriers to vaccine development include early age of RSV infection, capacity of RSV to evade innate immunity, failure of RSV-induced adaptive immunity to prevent reinfection, history of RSV vaccine-enhanced disease, and lack of an animal model fully permissive to human RSV infection. These biological challenges, safety concerns, and practical issues have significantly prolonged the RSV vaccine development process. One great advantage compared to other difficult viral vaccine targets is that passively administered neutralizing monoclonal antibody is known to protect infants from severe RSV disease. Therefore, the immunological goals for vaccine development are to induce effective neutralizing antibody to prevent infection and to avoid inducing T-cell response patterns associated with enhanced disease. Live-attenuated RSV and replication-competent chimeric viruses are in advanced clinical trials. Gene-based strategies, which can control the specificity and phenotypic properties of RSV-specific T-cell responses utilizing replication-defective vectors and which may improve on immunity from natural infection, are progressing through preclinical testing. Atomic level structural information on RSV envelope glycoproteins in complex with neutralizing antibodies is guiding design of new vaccine antigens that may be able to elicit RSV-specific antibody responses without induction of RSV-specific T-cell responses. These new technologies may allow development of vaccines that can protect against RSV-mediated disease in infants and establish a new immunological paradigm in the host to achieve more durable protection against reinfection.

Introduction

Respiratory syncytial virus (RSV) is a pneumovirus in the family *Paramyxoviridae*. RSV and metapneumovirus are the only two human pneumoviruses currently recognized, but there are several pneumoviruses of veterinary importance including bovine RSV, turkey rhinotracheitis virus, and pneumonia virus of mice. RSV was first described as chimpanzee coryza agent in 1956 (1) and associated with bronchiolitis in children in 1957 (2). There are two major genetic subtypes, A and B. These subtypes co-circulate, and the predominance of one over the other varies by year and geographic location (3).

Disease burden and populations at risk

RSV causes yearly wintertime epidemics of respiratory infection. It is the leading cause of hospitalization in U.S. children less than 1 year old (4) and one of the leading causes of clinic appointments in U.S. children less than 5 years of age (5). Globally, it is estimated that RSV causes >30 million lower respiratory tract infections each year resulting in >3 million hospitalizations, making it the most common cause of hospitalization in children under 5 years of age (6). Most children are infected during their first year of life, and all are infected by age 3 (7). Recurrent infections occur throughout life. In infancy the peak age of

hospitalization is ~2.5 months of age. Children with prematurity, bronchopulmonary dysplasia, and congenital heart disease are most susceptible to severe disease (8-9). Atopy or a family history of atopy has also been associated with severe disease in infancy (10-11). During childhood and adulthood, disease is milder but can be associated with lower airway disease and is commonly complicated by sinusitis (12). Disease severity increases in the institutionalized elderly, where it is estimated that RSV causes about ~12,000 deaths per year, about 1/3 the amount of excess mortality attributed to influenza (13-14). Severe disease also occurs in persons with severe combined immunodeficiency disease or following bone marrow or lung transplantation (15). Transplantation of other solid organs seems to pose less of a risk (16). Persons with immunoglobulin deficiencies or human immunodeficiency virus (HIV) infections do not experience severe disease, but HIV-infected children have been noted to shed virus asymptomatically for up to 200 days (17).

Factors associated with severe disease during primary infection

Lower respiratory tract involvement is common and associated with airway obstruction and hypoxia in infants, leading to respiratory distress and hospitalization. Several cohorts of children have been evaluated by single nucleotide polymorphism analysis (18). Those studies have found associations between disease severity and genes involved in allergic responses [interleukin-4 (IL-4) and IL-4 receptor], innate immunity (surfactant binding proteins), and inflammatory cytokines (e.g. IL-6, IL-8). Severe disease (hospitalization) is associated with childhood wheezing that lasts until teenage years. Analysis of respiratory secretions and pathology has also been done, attempting to associate the presence or absence of specific immune responses with disease severity. The histopathology of fatal cases of RSV suggests there is a relative paucity of $CD8^+$ T cells (19). Gender associations have been made linking males with more severe disease, and breastfeeding has been shown to provide a benefit only for girls (20). These findings suggest that modifications in the timing and composition of pre-existing vaccine-induced immune responses could have a significant impact on illness. There are also viral determinants of illness severity that have been less well defined. Virus load has been associated with the severity of clinical symptoms (21), and some epidemic years tend to result in more hospitalizations than others. However, this has not been clearly associated with new antigenic variants that would be expected to spread more easily in populations with less pre-existing immunity. There have also been minor sequence variations of surface glycoproteins associated with greater mucus production in animal models (22) or strain variation in the way Toll-like receptor 7 (TLR7) signaling pathways are activated (23), suggesting viral determinants may influence airway physiology and impact illness severity.

Major hurdles for vaccine development

Despite the importance of this viral pathogen, there are not adequate treatment or vaccine options available. Ribavirin is sometimes used in dire circumstances, but is not recommended in most cases. Passively administered antibody is licensed for use in neonates at highest risk for severe RSV disease. Its use has not been extended to the general population or adults, and it is only effective as prophylaxis, not therapeutically. Most of the infants hospitalized with severe RSV disease do not have identified risk factors. Active vaccination would be the ideal way of controlling this self-limited virus infection. Since RSV is not known to have an intermediate host or animal reservoir, it is possible that immunizing with a vaccine antigen before the first RSV infection could profoundly change the ecology of the virus in humans and change the immunological paradigm that allows RSV to continually reinfect humans. However, vaccine development for RSV faces several challenges unique to RSV: first is the young age of infection; second is the multiple mechanisms RSV uses to evade innate immunity; third is the lack of durable protective

immunity induced by natural infection; fourth, a legacy of vaccine-enhanced disease dating back to clinical trials done in the 1960s with a formalin-inactivated whole virus vaccine; and fifth, animal models do not faithfully replicate the pathogenesis of human RSV (Table 1).

Early age of infection

Severe disease in primary infection is best associated with underlying diseases that decrease the size and maturity of airways and increase pulmonary artery pressure. Severe infection occurs at a young age when both pulmonary and immunological development processes are incomplete. There is also genetic, immunological, and physiological evidence that co-existing allergic inflammation complicates RSV-induced disease. Lethal infection can occur when $CD8^+$ T cells are not available for viral clearance [severe combined immunodeficiency (SCID), bone marrow (BM) and lung transplant]. It is also known that passively administered neutralizing antibody can prevent disease without evidence of immunopathology. Therefore, the goal of vaccination should be to protect infants as early as possible with immune responses that include neutralizing antibody and $CD8^+$ T cells and that avoid allergic inflammation. Neonates immunized at or near birth would still have variable levels of maternal antibody. The level of neutralizing antibody available to protect the lower airway is the most important factor for prevention of severe disease or hospitalization. Infants with the lowest levels of maternal antibody will need the best and earliest vaccine-induced antibody response. While local mucosal antibody responses should be beneficial, serum antibody is sufficient to protect the lower respiratory tract where the gradient between blood and tissue is not as large as it is in the upper respiratory tract.

Evasion of innate immunity

RSV has evolved several mechanisms to modulate or evade pre-existing immune responses. RSV is typically a self-limited infection that is restricted almost exclusively to the respiratory epithelium, so it has a limited tropism and limited ability to spread or persist. Its lipid envelope, polymorphic geometry, and single-strand RNA genome make it relatively fragile and vulnerable in environments outside of the airway compartment. Despite these limiting features, a human population that is uniformly pre-immune, and lack of intermediate host or animal reservoir, it is highly effective at maintaining yearly epidemics. The primary strategy for RSV to maintain this successful niche appears to be organized around inhibiting Type I interferon (IFN) responses (24). The induction and effector functions of IFNs are central to innate immunity and serve important roles in the initial defense against viral infections. Two RSV nonstructural (NS) proteins, NS1 and NS2, are encoded at the beginning of the genome, signifying their importance. They collaborate to inhibit IFN-associated genes through INF regulatory factor 3 (IRF3) and signal transducer and activator of transcription 2 (Stat2) (25-26). The single-stranded RNA genome and double-stranded RNA replication intermediates from RSV should be potent inducers of signaling pathways through TLR or cytosolic RNA receptors. However, RSV has mechanisms to inhibit TLR signaling through both myeloid differentiation factor 88 (MyD88) and mitochondrial antiviral signaling protein (MAVS) (27), induce suppressor of cytokine signaling (SOCS) molecules (28-29) or interfere with RIG-I (30) to suppress signaling events intended to increase IFN production and effector function. The combined effect of these processes is particularly consequential for the neonate, whose innate immune responses are muted to begin with. It is possible that the gender-associated disease outcomes are related to the balance of these effects, because some of the gene products involved in regulating IFN expression and lymphocyte function [TLR7 and forkhead box protein 3 (FoxP3)] are encoded on the X chromosome. Interfering with these critical elements of innate immunity may be what allows RSV to successfully infect upper airway epithelium and spread to the lower respiratory tract before adaptive immunity can resolve the infection.

Failure to protect against reinfection

Although RSV is effectively cleared after primary infection and both antibody and T-cell responses are induced, RSV is able to reinfect people throughout life. Nearly all children are infected during the first two years of life, and about half of those infected as neonates are reinfected during their second year (31). Children and adults are infected every 3-10 years, and illness is manifest as an upper respiratory tract infection, often with sinus complications (8,12,32). Other viruses that can cause repeated infections are usually characterized by significant genetic and antigenic diversity. For example, HIV and influenza can reinfect as antigenic sites evolve, and people can be infected with distinct serotypes of rhinovirus or adenovirus. RSV is somewhat unique, because it commonly reinfects even though genetic diversity is not extreme and protective neutralizing antibody antigenic sites are highly conserved between strains. Naturally infected adults evaluated by pulmonary function studies have increased airway resistance for up to 8 weeks, indicating lower airway disease is common even when minimally symptomatic (33). One informative human challenge experiment showed that in healthy adults challenged every few months with the same strain of RSV, about 25% were infected each time and about half of those became symptomatic (32). These studies are now being reproduced with new human challenge stocks (34-35), and access to modern immunological techniques may provide some insight into the mechanisms of immune evasion. Most reinfection events are restricted to upper airway disease, unless subjects are immunocompromised. Therefore, the inability to prevent infection may be the consequence of a highly prevalent and contagious virus, effective evasion of local and innate immunity, and a steep gradient for transudation of antibody from serum to nasal epithelium. Mechanisms associated with evasion of local adaptive immunity have not been elucidated. Since repeated mucosal infections do not seem to be sufficient for protection against reinfection, another possibility is that the initial RSV infection permanently alters the characteristics of adaptive immune effectors and memory. There is evidence that RSV infection creates a sufficient antigenic stimulus to induce both antibody (36) and T-cell responses (37), but the durability of the antibody response is poor and the phenotype, functionality, localization, and durability of RSV-specific T-cell responses need additional investigation. Unlike RSV, measles virus (MV) infection typically induces life-long immunity. Although MV is a related paramyxovirus, its pathogenesis involves systemic spread from the initial site of inoculation in the respiratory tract. Protection has been associated with vanishingly low levels of serum antibody, which presumably prevents clinical disease by blocking virus dissemination in blood. Because RSV is restricted to the respiratory tract and does not have a viremic stage, it may require a higher level of systemic antibody or maintenance of local immunity to prevent or interrupt disease progression than MV. Since RSV replication and disease are restricted to the respiratory tract, one of the major questions for vaccine development is whether local mucosal immunity is required or whether systemically induced immunity would be sufficient.

Vaccine-enhanced disease

During the mid 20th century, there were a series of successful antiviral vaccines made by either inactivation or attenuation of wildtype virus. Methods of inactivation included Tween-ether, formalin, irradiation, or β-proprionolactone. Attenuation was accomplished by serial passage, cold-adaptation, or delivery by an alternative route. In this setting, shortly after the discovery of RSV, a series of studies was initiated to evaluate a formalin-inactivated RSV vaccine (FI-RSV) formulated with alum. Four studies were done in parallel in children of different age groups (38-41). The vaccine was delivered by intramuscular injection. In the youngest age cohort of children, less than 6 months of age, 31 were immunized with FI-RSV and 40 were immunized with a similar product derived from parainfluenza virus type 3 (41). During the next winter season, 80% of FI-RSV recipients required hospitalization and two children died. Only 2.5% of children in the control group required hospitalization. The

clinical disease in vaccinated infants was indistinguishable from typical RSV-mediated disease, but severe disease was much more frequent. The pathology in children who died demonstrated a pattern of inflammation centered around the small bronchioles typical of primary RSV infection. There was evidence of airway obstruction with fibrinous exudates that included sloughed epithelial cells, mononuclear cells, and an abundance of neutrophils and eosinophils. In one of the studies it was noted that blood eosinophilia was present in the vaccinees (38). Immunological assays available at that time showed high antibody titers measured by complement fixation, robust lymphoproliferative responses, and delayed-type hypersensitivity (DTH) (42). Subsequent studies with samples from these children showed relatively poor functional antibody responses with low neutralizing and low fusion-inhibiting activity (43-44). In addition, there was immunohistochemical evidence of immune complex deposition in small airways (45). There have been many attempts to model the FI-RSV vaccine-enhanced illness in animal models using mice (46), cotton rats (47), calves (48), and non-human primates (49-50). Considering the available data from animal models, the original FI-RSV vaccine trials, clinical trials of live RSV given parenterally or nasally without evidence of vaccine-enhanced disease, and studies associated various clinical and genetic factors associated with disease severity from primary infection, the following broad conclusion can be drawn. Vaccines that induce antibody responses with poor functional activity or induce $CD4^+$ T cells that produce IL-4 and are associated with eosinophilia should be avoided in RSV-naive neonates. This is highlighted and restated as one of the principles of RSV vaccine development listed in Table 2, that induction of the right antibody responses will be important for efficacy, and induction of the right type of T-cell responses will be important for vaccine safety.

Role of antibody in vaccine-enhanced disease

One of the few advantages available to RSV vaccine developers is the knowledge that passively administered antibody to a single epitope on the F glycoprotein with moderate neutralizing activity can protect high-risk infants from severe disease. This supports the earlier findings that the level of neutralizing activity passively transferred from mother to child (51) and passively administered polyclonal neutralizing antibody (52) to RSV are also protective against severe disease, primarily defined as hospitalization. These data strongly support the rationale for designing vaccines that induce neutralizing antibody. While immune-complex deposition was documented in children from the original FI-RSV vaccine trial and in animal models (45), this phenomenon has not been associated with severe disease from maternally derived or passively derived antibodies, and has not been associated with severe disease following reinfection with RSV. Also, unlike other vaccine-enhanced disease syndromes, in which antibody-dependent enhancement of disease occurs through facilitated entry by Fc-mediated binding, this has been evaluated in RSV and does not appear to apply (53). This phenomenon is typically associated with pathogens that have distinct macrophage tropism, which RSV does not. Therefore, designing vaccines that induce potent neutralizing antibody is a primary goal for vaccine development. There should be consideration given to durability of vaccine-induced antibody and its functional properties as titers wane. There are many parallels between the pathogenesis of the vaccine-enhanced illness associated with Tween-ether and formalin-inactivated measles vaccines and RSV (54). Atypical measles did not occur until several years after vaccination, as antibody titers waned below a certain threshold and T-cell responses associated with DTH and Type 2 cytokine production were maintained (55-56). The threshold of antibody needed for prevention of RSV infection is much higher than for measles, so more care should be given to the pattern of vaccine-induced T cells. As long as antibody levels are sufficient to prevent significant viral replication, the underlying T-cell response patterns may not have a major influence on disease manifestations. The findings of immune-complex deposition in small airways of the infants who died from FI-RSV vaccine-enhanced disease suggest that the

antibody and T-cell responses were not sufficient to prevent the accumulation of a large viral antigen load that was bound by RSV-specific antibody that could not neutralize the virus. This event presumably activated complement cascades and potentiated and modified the immune-mediated disease caused by the underlying infection.

The key reason that induction of neutralizing antibody should be the primary goal of vaccination is that the severity of disease is largely determined by the extent of viral replication. This is based not only on the direct destruction of epithelial cells but also on the level of immunopathology produced by innate and adaptive responses that can be directly related to the consequent antigen load. Antibody is the only adaptive immune effector that can prevent virus infection and thus is an optimal first line of defense for protecting airway epithelial cells and reducing antigen accumulation. If virus replication progresses or if the initial viral antigen inoculum is high enough to reach a certain threshold, there will be disease consequences. Therefore, it is important to consider the types of adaptive T-cell responses induced by vaccines, because they will serve as secondary lines of defense when antibodies and innate immunity is not sufficient prevent infection.

Role of T cells in vaccine-enhanced illness

The roles of T-cell responses associated with the FI-RSV vaccine-enhanced disease are controversial. These discussions have sometimes been confused by focus on selected endpoints or too much reliance on selected animal models. However, taken together, the accumulated data provide guidance for future vaccine development efforts. To determine which vaccine-induced T-cell responses would be most favorable, the major considerations should include virological, immunopathological, and developmental perspectives. There are three major reasons to avoid T-helper 2 (Th2)-associated responses with an RSV vaccine. First is the recognition of eosinophilia in the lung sections of infants who died in the original study. Second, there is a clear induction of Th2 immune responses in FI-RSV immunized animal models. Third is the epidemiological and genetic association of allergic inflammation and Type 2 immune effector polymorphisms with RSV-induced severe disease. However, this does not mean that all aspects of Th2-associated responses are necessarily bad. There is good evidence that eosinophilia and products from eosinophils have antiviral properties and can be associated with lower viral loads (57). Therefore, even though eosinophilia may be a surrogate marker for Th2 immune responses, the eosinophil may not be intrinsically harmful (58). Also, Th2-biased immune responses have been associated with antibody isotypes that have relatively low complement-binding capacity and with the induction of mucosal antibody responses, so there may be some potential advantages associated with the induction of Th2 responses from a virological perspective and from the standpoint of avoiding immune complexes and optimizing mucosal antibody production. However, based on immunopathology, the importance of effective viral clearance by $CD8^+$ T cells and the weight of clinical evidence, it is my opinion that Th2 responses should be avoided in RSV vaccine-induced immune responses. One reason is that animal models of FI-RSV vaccine-enhanced disease and associated Th2-biased responses consistently show increased severity of immunopathology using semi-quantitative histopathology scores. In addition, Type 2 cytokine responses are associated with increased mucus production, which is thought to contribute to the obstruction of small airways during RSV infection. Th2 responses are also associated with increased airway hyperresponsiveness that could potentiate the wheezing that is known to complicate severe RSV disease. Finally, the cytokines associated with Th2 immune responses, particularly IL-4 and IL-13, have been associated with diminished or altered $CD8^+$ cytolytic T-cell effector function and delayed viral clearance (59-63).

Clearance of RSV infection is accomplished largely by T-cell-mediated mechanisms (64). $CD8^+$ T cells are known to be important for clearance of RSV-infected cells in mice, in calves, and in humans. This role is supported by the observation that prolonged RSV

replication occurs in patients following allogeneic bone marrow and lung transplantation or persons with SCID syndrome. RSV infection in patients following solid organ or autologous BM transplantation is not as severe, suggesting that having major histocompatibility complex (MHC) class I alleles fully matched between lung epithelium and BM-derived immune precursors may have some importance for viral clearance. Although $CD8^+$ T cells can also cause immunopathology, this is in the context of a high magnitude T-cell response encountering a very high viral load (65) or large viral inoculum (66), which is unlikely to occur in the early stages of primary infection. Therefore, $CD8^+$ T cells should be favorable, if they can respond relatively early in the process of infection before the viral load has become too high and can clear virus-infected cells efficiently without excessive bystander pathology. In this sense, the $CD8^+$ T-cell response is analogous to the antibody response that can also create complications of immune complex deposition, if it occurs in the setting of a high virus load and has poor functional properties. The FI-RSV vaccine formulated with alum is unlikely to have induced $CD8^+$ T-cell responses. Immunization with live-attenuated RSV intranasally or live RSV intramuscularly (with the potential to induce RSV-specific $CD8^+$ T-cell responses) was not associated with vaccine-enhanced illness. Currently, there are no definitive contraindications for inducing these responses with future RSV vaccines. However, because $CD8^+$ T cells by nature cannot prevent infection, there is always some price to pay in terms of immunopathology for clearing virus-infected cells, and for an effective vaccine, the induction of $CD8^+$ T cells would have a lower priority than the induction of neutralizing antibodies. On balance, having vaccine-induced RSV-specific $CD8^+$ T cells will provide an advantage to the host. In addition to their potential contribution to early viral clearance, $CD8^+$ T cells (67) and NK cells (68) have been associated with diminished Th2 responses and eosinophilia in murine models of RSV, although the mechanisms are not fully elucidated (69-70).

Animal models

Although human RSV (hRSV) is highly restricted to the respiratory tract *in vivo*, it is able to infect a wide variety of cells *in vitro*, particularly adherent cells. Much of the initial attachment process involves binding to glycosaminoglycans (GAGs), particularly those containing iduronic acid such as heparan sulfate (71), or to C-type lectins such as surfactant proteins (72). GAGs and C-type lectins are abundantly expressed on many cell types across many species. In that sense, it is surprising that fully permissive animal models for hRSV have not been identified, suggesting that there may be other receptor binding events that are needed for efficient entry and that restrict tropism. It may also suggest that the mechanisms hRSV uses for inhibiting Type I IFN induction and effector functions are highly adapted for evading innate immunity in humans and may explain why hRSV does not have a known animal reservoir or intermediate host.

Nonhuman primates

Human RSV was first recognized as chimpanzee coryza agent and is able to infect chimpanzees. While disease manifestations in hRSV-infected chimpanzees are not extreme, they have been effectively used to evaluate live-attenuated RSV vaccine candidates, and infection of chimpanzees is a good method for rank-ordering viruses for the level of attenuation for replication in the upper respiratory tract (73). Multiple other nonhuman primate (NHP) species have been evaluated for susceptibility to hRSV infection but are semi-permissive. African green monkeys (AGMs) have been the most extensively studied. After a combined nasal and intratracheal inoculation peak titers of $>10^5$ and $>10^3$ pfu/ml can be achieved in lung and nasal secretions, respectively, and virus is shed for ~10-12 days (74). AGMs have been used to model the FI-RSV vaccine-enhanced illness and have demonstrated enhanced pathology (49). Eosinophilia and type 2 cytokine production have been seen in FI-RSV immunized rhesus macaques, but the RSV titers in untreated macaques

are relatively low and are typically measured by polymerase chain reaction (PCR) (50). Therefore, currently available NHP models of RSV are not sufficiently permissive to use them as a gatekeeper for either efficacy or safety, although they may be useful for rank-ordering potency and making 'no go' product development decisions.

Rodents

Human RSV has been extensively studied in rodents, particularly cotton rats (*Sigmodon hispidis*) (75) and mice (76). Rodent models have been useful for a number of purposes but also have significant limitations. Cotton rats have been used to evaluate RSV therapeutics and pathogenesis. They provided much of the key data that led to the licensure of passive antibody prophylaxis of RSV in premature infants (77). Mice have been used to study various aspects of pathogenesis, particularly the immune response patterns induced by prior immunization and influenced by allergic inflammation (76). Guinea pigs have also been effectively used to investigate RSV, particularly for questions about allergic inflammation and airway physiology (78), but will not be discussed further here. Both cotton rats and mice are semi-permissive for hRSV replication. Challenge inocula above 10^5 pfu are required for productive infection, above 10^6 pfu for significant lung pathology, and close to 10^7 pfu for induction of clinically apparent illness. Using a large volume, high magnitude inoculation to initiate infection does not recapitulate the progression of RSV infection from upper to lower airway that occurs in humans. This has two important consequences if using rodent models to predict the extent of disease that may be seen in humans. One is the pace or temporal progression of infection. The other is the size of the viral antigen load that is presented to the lower airway at the time of initial exposure to virus. Human infection is more likely to involve a relatively small viral inoculum in the upper airway that is amplified by a few rounds of local replication followed by progression to the lower airway by a combination of cell-to-cell spread and aspiration of respiratory secretions. The initial period of local replication provides an opportunity for adaptive immune responses with both effector and regulatory functions to be initiated prior to large viral loads accumulating in the lower respiratory tract. The high titer inoculum given directly into the lower airways not only bypasses this 2-4 day interval and the consequent modulatory effects of innate and early adaptive responses, but forces the innate immune defenses of the lower respiratory tract to deal with a significant antigen load from defective viral particles as well as the replication competent particles measured by plaque assay. For RSV this could be several logs higher than the titered stock because of the proclivity for producing defective particles. The consequences on early innate responses and the timing of adaptive responses is therefore a major consideration when interpreting the findings in rodent models.

In rodents, the small airway epithelium is not as extensively infected as it is in humans, and most virus replication occurs in Type 2 alveolar pneumocytes. More airway epithelium is involved if there are significant deficiencies of Type 1 IFN function either by removing inductive or effector pathways. For example, in mice with combined MyD88 and MAVS gene knockout, there is extensive infection and viral antigen accumulation in the bronchiolar epithelium (27). This finding suggests that the multiple mechanisms RSV employs to evade early Type I IFN responses are not fully effective in rodents and demonstrates that these models are missing a key determinant of RSV pathogenesis in humans.

The pathogenesis and severity of RSV disease in humans is highly variable, ranging from subclinical to fatal. While RSV infects all children, only 2-5% require hospitalization. Therefore, using a single strain of inbred mice or even small groups of outbred rodents is going to model a small fraction of the potential responses within a diverse human population. For example, BALB/c mice have been a popular model for studying RSV-induced immune responses. They make a highly dominant K^d-restricted CD8 response to an epitope in M2 ($M2_{82-90}$) (79) and an IA^d-restricted CD4 response to an epitope in G

($G_{184-198}$) (80). These responses utilize a relatively restricted TCR repertoire, Vβ8.2 (TRBV 13.2) (our unpublished data) and Vβ14 (81), respectively, and have distinctive phenotypic and functional profiles. The K^dM2_{82-90} response has an extreme effector phenotype, produces large quantities of IFN-γ, immunodominates other T-cell responses, and can produce immunopathology when present at high frequencies. The $IA^dG_{184-198}$ response produces IL-4 and IL-13 in addition to IFN-γ and is associated with allergic inflammation and airway hyperresponsiveness (82). Studying how the contribution of these two competing T-cell responses impacts RSV-mediated disease pathogenesis and influences airway physiology has been very informative. These studies have guided many of the current hypotheses about the basis for FI-RSV vaccine-enhanced illness and the mechanisms of RSV-induced airway hyperreactivity. However, the pathogenesis of hRSV in BALB/c mice, which is largely determined by the balance of these two distinctive T-cell responses, is not sufficient for anticipating the spectrum of clinical responses likely to occur in diverse human populations.

Rodent models of RSV have value in understanding interaction of allergic inflammation and virus-induced immune responses and additional value for understanding how immune responses are coordinated and regulated. They have faithfully predicted the efficacy of passively administered antibody and antivirals developed for RSV. They can also provide some evidence of the patterns of immune responses that may be seen in the setting of prior immunization. They are useful for rank ordering the immunogenicity of vaccines. While rodent models of hRSV are useful for negative selection of vaccine candidates, they are limited in their ability to positively select and discriminate candidate vaccines going forward. For example, if a vaccine concept does not protect in a rodent model of hRSV, its efficacy would not be adequate for advancing into human studies. Likewise, if a vaccine candidate induced IL-4 production, eosinophilia, and other indications of Th2 response in rodent lungs post-challenge with RSV, its safety for use in seronegative neonates would be questioned. However, protection of rodents and lack of Th2 responses or enhanced disease in rodents does not guarantee that the vaccine will achieve efficacy and safety in human neonates. Therefore, rodent models should be part of the preclinical assessment of candidate RSV vaccines but need to be interpreted conservatively.

Models utilizing nonhuman pneumoviruses

There are selected animal models using pneumoviruses other than human RSV that have advanced our understanding of pneumovirus pathogenesis in general and supported vaccine development concepts. Investigations of bovine RSV (bRSV) in calves have been particularly informative in demonstrating the key features of FI-RSV vaccine-enhanced illness and in the development of effective veterinary vaccines (48,83). Bovine RSV has also been studied in infant lambs for which systems have been established to do extensive measurements of airway physiology (84). This model may have particular value in better understanding mechanisms of RSV-induced apnea (85). The pathogenesis of pneumonia virus of mice (PVM) has been explored in murine studies, and mice have also been used to evaluate potential RSV vaccine concepts (86). These model systems have special value, because they utilize viruses that are matched to the appropriate host. The mutual adaptations that occur during the co-evolution of virus and host create a unique sequence of molecular and cellular events that cannot be faithfully captured in model systems in which the virus is not matched with it natural host. Studying viruses in their native hosts is more likely to authentically reflect the pathogenesis of natural infection. However, because these viruses are not hRSV, the models can only be used to evaluate vaccine concepts and cannot be used to study the immunogenicity, dose effects, or safety of a vaccine developed for humans. For that reason and because of space limitations, more detailed descriptions of these important model systems are not attempted here.

Options for RSV vaccine development

The expansion of vaccine technologies over the last two decades has provided new tools and knowledge that can be applied to RSV vaccine development. Here we consider subunit, live attenuated viruses, and gene-based vector approaches. Whole inactivated virus, no matter how it is adjuvanted, is going to face major regulatory hurdles in RSV-seronegative infants and will not be discussed as an option.

To achieve vaccine-induced protective immunity, effector mechanisms should be present early after exposure to mitigate the viral evasion of innate immune mechanisms. This would be best accomplished by the presence of neutralizing antibody. In addition, if effector T cells were present more rapidly after infection, viral clearance would potentially be accomplished earlier. This would result in less pathology, because fewer host cells would be lost and the total viral antigen load would remain lower and be less likely to induce excessive T-cell responses that could interfere with gas exchange and damage tissue. Presumably, this would result in less illness during the infection, less repair, and fewer long-term consequences for airway architecture and function.

Clinical development

There are several potential goals for RSV vaccine development, and the characteristics of the vaccine and study designs will vary depending on target population. The ideal and ultimate goal for a RSV vaccine would be to protect neonates from infection during the first season and eliminate the large burden of hospitalization that occurs in the 2-3-month-old age group. The only immunization strategy that has been evaluated in 1-2 month-old infants utilized live attenuated virus. Virus attenuation was achieved by cold-adaptation and chemical mutagenesis and ultimately by transferring selected mutations into infectious molecular clones. Additional modifications were empirically derived by deleting selected nonessential genes (NS1, NS2, M2-2, or SH) or adding additional attenuating mutations not necessarily associated with temperature sensitivity (87). These live viruses were evaluated in immunodeficient mice and chimpanzees to achieve a rank order of attenuation, then tested in adults, seropositive children (>2 years of age), and seronegative children, before evaluation in infants (<6 months of age). Two vaccines have been evaluated in seronegative infants. The first (cpts248/404) caused a high frequency of nasal congestion and was considered to be insufficiently attenuated (88). Nasal congestion was counted if it interfered with eating, sleeping, or resulted in obligate mouth-breathing. The other virus (rA2cpts248/404/1030/ΔSH) was evaluated in 32 infants at one of two doses ($10^{4.3}$ pfu or $10^{5.3}$ pfu), and 12 placebo recipients (89). The low and high dose inoculations resulted in 63% and 94% infection rates with 2.4 $\log_{10}$pfu/ml and 3.5 $\log_{10}$pfu/ml peak titers in nasal secretions, respectively. This was about 2 $\log_{10}$ lower than cpts248/404 and reduced the frequency of nasal congestion to 19% and 44% for the 2 dose levels tested. The most striking result of this study was that the frequency of virus shedding after a second vaccine inoculation 4-8 weeks later was only 29% and 44% for the low and high doses with much lower peak virus titers. While there was evidence of infrequent F- and G-specific IgA antibody responses in immunized infants, they were not sufficient to correlate with protection. Live-attenuated virus vaccine approaches are discussed in more detail below.

Protecting infants from 6 months to 2 years of age from their first or second RSV infection could also have a major impact on morbidity. There is a high attack rate in this age group and RSV remains a common cause of hospitalization and doctor visits up to the age of 5. Some of the advantages of focusing on this age group include: (i) waning of maternal antibody to RSV or delivery vehicles; (ii) they are past the stage of idiosyncratic apnea events; (iii) maturation of immune system including capacity for somatic mutation and development of high affinity antibodies has occurred; (iv) children in this age range are a

common source of RSV transmission to younger infants and parents; and (v) lower frequency of breastfeeding makes nasal delivery more feasible.

Immunization of persons older than 2 years of age implies that vaccination will be done in the setting of pre-existing immunity, since nearly everyone has been infected by that age. Children and adolescents who have been infected multiple times with RSV with diminishing clinical symptoms are less likely to benefit from RSV immunization than infants. However, there are two major subgroups of adults that should be considered as target populations for RSV immunization. First are pregnant women or young women expecting to become pregnant. Immunization of pregnant women, particularly those with deliveries anticipated in the last quarter of the calendar year, could potentially boost antibody levels and improve the effectiveness of passively transferred immunity in the newborn. Immunization of pregnant women is controversial and is a complex topic that cannot be adequately covered here. Vaccinating women of child-bearing age prior to pregnancy is a potential option but would require vaccines that could induce a durable boost of pre-existing antibody levels. One of the major biological hurdles for immunizing young women involves the property of RSV to repeatedly infect healthy adults. Vaccination would have to be significantly better than natural infection in terms of magnitude and durability of immune responses to have a measurable difference on levels of maternally derived RSV-specific antibody.

Adults >65 years of age represent another target population that should be considered in RSV vaccine development. Immunization of elderly persons faces the problems imposed by pre-existing immunity described above and has the added challenge of the aging immune system. While RSV does influence morbidity and mortality in the institutionalized elderly, the pathogenesis is different than the bronchiolitis of primary infection and invariably involves underlying cardiopulmonary dysfunction. In that sense, RSV may be more of a triggering event than the direct cause of the pathology leading to illness in this age group. This would potentially make the threshold for protective immunity more difficult to achieve. There are several laboratory challenges for conducting RSV vaccine studies in adults. One is that everyone has pre-existing immunity, so all immunogenicity measurements will have to be compared to background levels. This significantly complicates the identification of immune correlates. Secondly, the diagnosis of RSV in adults is more difficult than in children experiencing primary infection. Rapid antigen detection and culture are less likely to be positive in RSV-infected adults, so the infection endpoint requires nucleic acid detection by PCR (90).

Antigen selection

Antigen selection for a RSV vaccine, as for any viral vaccine, requires consideration of the protective antigenic sites, antigenic diversity, genetic variability, and the immunological effectors targeted for induction, toxicity, or immunomodulatory properties, and feasibility of production or expression. For live-attenuated virus vaccines, in which most if not all antigens are included, it is still important to know how attenuation has affected expression of the desired antigenic determinants and avoided expression of antigens that may cause toxicity or adversely impact vaccine-induced immune responses. If RSV antigens are produced from gene-based vectors, the fact that RSV has an exclusively cytoplasmic replication cycle is important to consider. The native genes have never had to adapt to the nuclear environment. If the delivery vehicle initiates transcription in the nucleus like DNA plasmids or recombinant adenovirus vectors, the RSV gene will have to be extensively codon-modified or transcripts will be rapidly degraded. If the vector has a cytoplasmic replication program like alphavirus or poxvirus vectors, codon modification is less critical.

RSV F is a trimer of 70 kDa heterodimers. It is a type I integral membrane glycoprotein and mediates pH-independent membrane fusion from without during viral entry and cell-to-cell

spread. RSV F is similar to HIV-1 gp160, ebola GP, and other paramyxovirus fusion protein that require furin cleavage to expose a hydrophobic peptide and assume the fusion-active conformation (91). The RSV F glycoprotein is surface-expressed and has at least three identified major antigenic sites associated with neutralizing antibodies (92). The known neutralizing epitopes in RSV F are all located in the region between the 2 heptad repeats (HR) in the major cleavage fragment F1. One of these sites is the epitope for palivizumab, a licensed monoclonal antibody (mAb) that significantly reduces RSV-associated hospitalizations when passively administered to infants at risk for severe disease (93). Therefore, F is a known protective antigen and a target for neutralizing antibody and is considered the most important antigen for including in a RSV vaccine. F is highly conserved between strains, even between strains from the A and B subtypes. F-specific neutralizing antibodies to antigenic sites A (~aa255-275) and C (~aa422-438) are cross-protective. RSV F is unique among fusion proteins, because it has two furin cleavage sites that liberate a 27 amino acid peptide containing 2 of the 5 N-linked glycosylation sites. The biological functions of this peptide have not been fully defined, although a homologous peptide from bRSV (termed virokinin) can interact with tachykinin receptors and cause smooth muscle contraction (94). Like G, RSV F also has heparin-binding domains (95). In addition, F has been shown to interact with the TLR4/CD14 complex and can induce IL-6 production in monocytes (96). The potential biological effects of the 27 aa peptide, heparin-binding, TLR interaction, and other biochemical aspects of RSV F need to be considered in vaccine design and evaluation.

The RSV G glycoprotein is a 90 kDa type II integral membrane protein that has 4 N-linked glycosylation sites and is also heavily O-glycosylated. RSV G has been associated with viral attachment and is sometimes referred to as the attachment glycoprotein. It is also the target of known neutralizing antibodies, and G is the most variable of RSV proteins, suggesting that it is under immune pressure (91). Variability in the G glycoprotein is the major distinction between the major RSV subtypes. For these reasons, G is a candidate antigen to be considered in vaccine design. Interestingly, G-deleted viruses can still infect cells *in vitro*, indicating that G is not required for attachment and entry (97), although the absence of G does diminish infectivity *in vivo*. This suggests a potential role for G in immune evasion or immunomodulation, which should be considered in vaccine design. RSV G has a chemical composition consisting of ~30% serines and threonines and ~10% pralines, which is unusual for viral glycoproteins. The closest counterpart is the ebola GP, which has a mucin-like domain that appears to cap the receptor binding domain prior to pH dependent cleavage by cathepsin L (98). RSV G has an alternative initiation codon at amino acid 48 in the transmembrane domain, which results in significant shedding of soluble G from infected cells (99). The soluble form of G is thought to function as a decoy for neutralizing antibody (100). The mucin-like domains are located on both ends of the protein and around a nonglycosylated central domain that has 4 conserved cysteines organized as a cysteine noose followed by a heparin-binding motif (101-102). A ‘CXXXC’ sequence can interact with the fracktalkine receptor (CX3CR1) (103), and the propensity for G binding to glycosaminoglycans and C-type lectins provide multiple potential mechanisms for G to affect immune functions. Decisions to include G as a vaccine antigen merit additional investigation into the potential biological effects RSV may have on the immune response.

Internal (non-surface exposed) antigens are highly conserved between RSV strains and are known to be a source of many T-cell epitopes (104-105). $CD8^+$ T-cell responses are thought to be important for RSV clearance because of the severe and progressive disease that occurs in patients with selected immunodeficiency syndromes and supportive data from animal models. $CD8^+$ T cells also have favorable immunomodulatory properties (67,70). While certain $CD4^+$ T-cell responses have been associated with vaccine-enhanced disease, they have value for amplifying antibody responses and other T-cell effector mechanisms.

Therefore, in gene-based vectors including chimeric viruses, if a broader set of T-cell responses is desired, proteins such as N, M, or M2 could be considered as additional vaccine antigens, in combination with F and G. The SH (small hydrophobic) protein of RSV has surface exposure and is thought to possibly function as pentameric ion channel (106). Prior efforts to immunize with this protein have not successfully induced neutralizing antibody and because of its limited size does not contain many potential T-cell epitopes. Therefore, it has not been considered to be a high priority vaccine antigen. The NS1 and NS2 (nonstructural) proteins have significant immunomodulatory activity and, even though they have been viewed as potential therapeutic targets, have not been strongly considered as vaccine antigens. The large polymerase protein (L) and phosphoprotein (P) have not been studied carefully for their potential value as vaccine antigens.

Vaccine delivery platforms

Vaccine antigens have traditionally been delivered by infection with attenuated live virus or injection of whole inactivated virus. Fifteen of the 17 licensed antiviral vaccines fall into one of those categories, and the other two are virus-like particles (VLPs). The successful development of VLPs for hepatitis B (HepB) and human papillomavirus (HPV) belies the slow pace in achieving new licensed vaccines based on the significant advances in molecular biology over the last 30 years. It is likely that vaccines for RSV, and other viral pathogens with difficult biological properties like HIV and herpes simplex virus, will require the application of new technology platforms, in addition to a greater depth of understanding disease pathogenesis and mechanisms of immunity (107).

Live-attenuated viruses and vectors

Although whole inactivated RSV would be difficult to advance again into clinical evaluation because of safety concerns, vaccination with live attenuated RSV given parenterally or mucosally can be done safely. Early trials of live RSV given intramuscularly were unsuccessful because of poor immunogenicity but were not associated with signs of aberrant immune responses or enhanced illness. Therefore, much effort has gone into the development of live attenuated candidate RSV vaccines. The leading candidate viruses are based on attenuating mutations that cause temperature sensitivity discovered during *in vitro* cold-adaptation. The development of a system to construct infectious molecular clones of RSV (108) has allowed the introduction of these and other selected mutations into precisely engineered constructs and the production of highly characterized attenuated vaccine strains. Some of these viruses have been evaluated in seronegative infants (1-2 months of age) and have been shown to partially protect against a second dose of the vaccine strain as noted above. This approach has the advantage of utilizing most of the antigenic content of RSV, and the proteins should be expressed in their native conformations. Since it is delivered nasally, it should induce local immunity in the respiratory tract, which may be important for protection against RSV. A live attenuated molecular clone (MEDI-559) is now in Phase II clinical evaluation in children (5-24 months of age) and infants (1-3 months of age). The live attenuated virus approach has the following challenges, particularly if it is targeted for neonates prior to their first infection. (i) The virus needs to be sufficiently attenuated to avoid significant disease from the primary infection caused by vaccination and retain sufficient replication capacity to generate a protective antigen exposure. Nasal congestion is problematic in infants during breast- or bottle-feeding. (ii) The therapeutic window (between the thresholds for efficacy and symptoms) needs to be achieved in a large majority of infants, erring on the side of safety. This is challenging because of the wide variations in developmental status, levels of transferred maternal antibody, MHC alleles, and innate susceptibility to infection among infants under 2 months of age. It will also be difficult for an attenuated virus to achieve better immunity than infection with wildtype virus, which does not provide solid protection from reinfection. (iii) Idiosyncratic reactions, particularly

apnea, in this age group may be associated with a vaccine delivered in the airway whether perceived or real. (iv) RSV is difficult to grow to high titer, has a relatively high ratio of defective to replication-competent particles, is relatively fragile to freeze-thaw, and vulnerable under most storage conditions. These features will complicate the manufacturing, lot-to-lot consistency, and distribution of a live-attenuated RSV vaccine. Delivery of live RSV intramuscularly is another approach to attenuation and avoids many of the challenges listed above. However, the issues noted under point (iv) would still apply, and the earlier failure of live virus delivered parenterally may have been in part due to the low dose levels achieved. The ultimate attenuation would be a replication-defective RSV that can only express viral antigens from the initial transduced cell.

Chimeric viruses and replication-competent gene-based vectors

An approach that retains the advantage of replication in the respiratory tract but avoids most of the noted challenges is the use of chimeric viruses. Selected relevant genes from RSV have been expressed in related paramyxoviruses like bovine parainfluenza (PIV), Newcastle disease virus (NDV), or Sendai virus (SeV) that can be delivered via the respiratory tract. These viruses are attenuated because their native tropism is not human. Therefore, replication is limited *in vivo*, but *in vitro* replication properties are more favorable than RSV for manufacturing. Also, the frequency of pre-existing immunity against these viruses is very low, so the impact of maternally derived antibody will be negligible. Another version of this approach is to express RSV antigens from replication-competent vectors like adenovirus serotype 4 (Ad4), vesicular stomatitis virus (VSV), or BCG, for either mucosal or parenteral delivery. Each of these vectors has unique properties that may provide advantages or challenges depending on the target age group, vaccine antigen, and goals of immunization, but space limitations do not allow a full discussion here. Bovine PIV3 expressing the RSV F and the hPIV F (MEDI-534) is the only candidate product in this category that has advanced to clinical trials. Data have only been reported for seropositive children in whom it appeared to be safe. However, there was no viral shedding and no significant immunogenicity. This construct replicated to relatively high titers in RSV-naive AGMs (~5.5 and ~7 $\log_{10}$pfu/ml in nasal secretions and BAL, respectively) and protected them from challenge with wildtype RSV, even though serum neutralizing antibody titers remained relatively low (~1:16) (74).

Replication-defective gene-based vectors

There are a number of vector systems derived from nucleic acids, viruses, or bacteria that can deliver a gene encoding the vaccine antigen of choice. These systems all have nuances involving promoters, tropism, delivery options, durability of expression, gene insert size, immunostimulatory properties, vector-specific immunity, and methods of manufacturing that are important to consider. Vector systems of note in this category including adeno-associated virus (AAV), vesicular stomatitis virus, herpesviruses, poxviruses, naked DNA, and encapsidated DNA will not be discussed because of space limitations. Alphavirus vectors will be discussed briefly, and recombinant adenovirus vectors (rAd) will be discussed in more depth. Replication-defective vectors need to have robust manufacturing capacity, because achieving sufficient antigen expression for immunization depends on the vaccination dose. In most cases, delivery is parenteral, because it is difficult to achieve enough antigen production from a single round of expression when delivered mucosally. A major advantage of this approach is that gene expression should mimic natural infection resulting in authentic protein structures to elicit relevant antibody responses and proteasomal processing and MHC class I antigen presentation for $CD8^+$ T-cell induction. Other advantages of this approach include the improved safety profile because of lack of replication capacity, avoidance of immune evasion strategies by selective gene expression,

targeted antigen delivery based on selected vector tropism, and the ability to select vectors with low seroprevalence to avoid pre-existing anti-vector immunity.

Alphavirus vectors based on Venezuelan equine encephalitis virus (VEE), Semliki forest virus (SFV), or Sindbis virus have all been successfully used to express vaccine antigens (109). They are particularly attractive as vector systems because of the self-amplifying RNA replicon that has the potential to significantly increase antigen expression levels. They merit special consideration for RSV vaccine development, because they have an entirely cytoplasmic transcription program, which means codon-modification requirements will be less restrictive. In addition, murine experiments showed that parenteral immunization with recombinant VEE could uniquely elicit mucosal IgA responses to the vaccine antigen (110), which would be relevant to the airway-restricted RSV. VEE vectors expressing RSV F have been evaluated in mice and cotton rats and demonstrated immunogenicity, protection, and immune response patterns that do not include Th2-like responses (111). Therefore, this platform technology is a candidate for clinical evaluation. The major limitations of this vector platform have been manufacturing capacity and translation of immunogenicity profiles from mice to primates.

rAd, particularly serotype 5, expressing HIV envelope glycoproteins have been extensively studied in clinical trials and have been shown to be well tolerated and immunogenic (112-114). The rAd5 vector was initially designed for gene therapy, but because of induction of vector-specific immune responses and rapid clearance, its value as a vaccine exceeded its usefulness for gene therapy. One of the most attractive features of the rAd vector platform is the robust manufacturing capacity with yields ranging above 10^{13} particle units from a single 10 liter bioreactor production run. There are 51 recognized serotypes of human adenoviruses divided into 6 species (A-F). Ad5 is a species C virus and uses the coxsackievirus adenovirus receptor (CAR) through binding to Fiber and an integrin co-receptor that binds an ‘RGD’ sequence on the Penton base (115). In humans, CAR is widely expressed in gap junctions between epithelial cells and myocytes and is also expressed on human erythrocytes (116). The biology underlying the reason rAd5 is more immunogenic for both induction of antibody and $CD8^+$ T-cell responses than other rAd vectors has not been explained, but is the subject of intensive investigation because of 3 specific concerns associated with rAd5. The first is a historical association with a highly publicized adverse outcome in a gene therapy study (117). This was a case in which an extremely high dose of rAd5 was injected intravenously directly into the liver of a person with abnormal liver function. The extreme antigen load led to an acute inflammatory response, thought to be related to an antigen-induced ‘cytokine storm’, and the death of the patient. While this case was tragic and a valid reason for caution, it is not directly relevant to the use of modest doses of a vaccine vector given intramuscularly, and subsequent clinical trials of candidate rAd5 vaccines have been conducted without serious adverse events related to vaccine-induced inflammation. A second concern arose following the STEP trial (118) in which a rAd5 vector expressing the Gag, Pol, and Nef genes from HIV-1 was evaluated in individuals at high-risk of HIV-1 infection. In this study, among MSMs (men who have sex with men), vaccinees had a higher rate of HIV-1 infection than placebo recipients. The higher infection rate was associated with men who were uncircumcised and tended to be greater in those with pre-existing immunity to Ad5. While the basis for vaccine-induced increased infection has not been fully explained, this outcome has created a stigma for rAd5 that has been difficult to overcome, although it is not relevant to the purpose of protecting infants from RSV. The third major limitation for rAd5 is the relatively high Ad5 seroprevalence in the general population which is ~50% in North America and can be >90% in some developing countries (119-120). Pre-existing immunity to rAd vectors has been shown to diminish the magnitude and frequency of T-cell responses to the vaccine antigen and to a lesser extent blunts the vector-induced antibody responses (112,121). Therefore, the

rAd5 vector platform for adult populations may have diminished efficacy because of pre-existing Ad5 immunity. The effect of pre-existing immunity can be mitigated by priming immunizations, so rAd5 could still be considered as a booster in a heterologous vector combination regimen. In the case of RSV, there may be a window of opportunity to use rAd5 vectors. Children between 6 months (after maternally derived antibody has waned) and 2 years of age are Ad5-seronegative (120). Therefore, targeting that age group with rAd5 vectors expressing RSV antigens would be most advantageous.

Alternative rAd vectors have been constructed using species B (serotypes 14 and 35) and D (serotypes 26 and 28) human adenoviruses (122), chimeric adenoviruses in which fiber genes or immunogenic domains from hexon are swapped with rare serotype viruses (123), and from adenoviruses derived from nonhuman primates (124). These vectors have been developed primarily because of their relatively low seroprevalence and engineered to be as immunogenic as possible. For immunization against RSV in the presence of maternal antibody (under the age of 4 months) these rare serotype rAd vectors may have advantages over rAd5. Since adenovirus genes are expressed in the nucleus, the sequence of RSV genes inserted into any rAd vector will need to be carefully codon-optimized to ensure optimal expression.

Subunit proteins

Immunization with a single purified protein is one of the simplest possible vaccine platforms. However, except in the case of HepB surface antigen or HPV L1 that both assemble into VLPs, subunit vaccines have not been successfully licensed for any viral vaccine product. Purified RSV F glycoprotein (PFP-2) formulated in alum has been evaluated in clinical trials in adults. It was found to be well tolerated and modestly immunogenic. A single injection of 50 μg in alum in pregnant women at 30-34 weeks of gestation only induced a >4-fold rise in serum neutralizing activity in ~10%, but antibody to F in babies and in breast milk was significantly higher among the vaccinees (125). A single dose of this same vaccine in healthy elderly subjects (>60 years of age) elicited >4-fold rise in serum neutralizing activity against either RSV A or B subtype in 61% (126) and in frail elderly in 47% that was relatively stable for about 6 months. Another product consisting of a mixture of F, G, and M was also tested in subjects >65 years of age. In this study, single doses of 25, 50, and 100 μg were given with alum or a 100 μg dose was given without alum. Surprisingly, the non-adjuvanted product induced the highest antibody responses, and 58% developed a >4-fold increase in serum neutralizing activity (127). It is intriguing to consider the possibility that this antigen assumed some VLP structures, since it has been shown that matrix (M) protein from other paramyxoviruses is sufficient to form VLPs (128). Chimeric VLPs based on the Newcastle disease virus M and containing RSV G have been shown to be immunogenic in mice (129). Another subunit product based on the central conserved region the G glycoprotein (aa130-230) was constructed as a fusion protein with an albumin-binding domain from streptococcal protein G, produced in prokaryotic cells and formulated with an alum-based adjuvant (Adjuphos). Despite promising results in murine studies, studies in adults showed relatively low capacity for inducing neutralizing activity (130), and challenge studies in rhesus macaques showed no reduction of viral load, vaccine antigen-specific induction of IL-13, and detectable eosinophilia in lung (131).

Because of the legacy of vaccine-enhanced disease associated with FI-RSV, there are significant regulatory hurdles for advancing subunit proteins into seronegative infants. Given as a protein, even with modern adjuvants, subunit protein vaccines will elicit primarily $CD4^+$ T-cell and antibody responses. Relative to live attenuated virus or gene-based vectors, there will not be a significant $CD8^+$ T-cell response. Not having the positive influences of viral clearance and modulation of $CD4^+$ T cells away from Th2 $CD4^+$ T-cell responses provided by $CD8^+$ T-cell responses creates additional theoretical concerns about

the potential for vaccine-enhanced disease when immunizing RSV-naive infants. These concerns would have to be weighed against the quality, magnitude, and durability of the neutralizing antibody induced by the candidate subunit product. It is possible that tertiary and quaternary structural features of the proteins or the immunostimulatory properties of a VLP are necessary to achieve the specificity, breadth, and magnitude needed for protective antiviral responses. The RSV F glycoprotein is thought to exist as a trimer on the surface of the virus like other class I fusion proteins. Preservation of the antigen sites that are targeted by neutralizing antibodies, the oligomeric state, and other structural aspects of F or G in addition to adjuvant selection should be thoughtfully considered if they are produced as purified proteins for candidate vaccines.

Recently, the structures of the epitope (aa254-277) recognized by palivizumab and motavizumab (132) and another epitope (427-437) recognized by the antibody 101F (McLellan *et al*., manuscript submitted) have been solved. These data suggest that even more limited subunit vaccines could be envisioned, expressing just the structures of key neutralizing epitopes. Building distinct epitope structures onto other protein scaffolds has been developed conceptually (133) and piloted in HIV vaccine design with the gp41 2F5 epitope (Ofek *et al*., manuscript submitted). This structure was able to elicit structure-specific antibody, but the antibody was unable to neutralize HIV-1 isolates. One potential derivative of producing a scaffolded epitope is that a vaccine antigen could be constructed that induced antigen-specific antibody against the structure without inducing antigen-specific T-cell responses since the linear sequence required for a T-cell epitope is not required for recreating the structure. Interestingly, palivizumab, motavizumab, and 101F antibodies bind trimeric F protein with much higher affinity than they bind peptide, even though they are described as linear epitopes. This and historical data from other peptide immunization studies suggest that the determinants for antibody recognition and binding of an epitope are more complex and include a greater surface area than just that between the linear peptide and the combining region of the antibody. Therefore, the concept of building a vaccine response one epitope at a time will require new breakthroughs in our understanding of what defines an epitope and the structural basis of antibody-epitope affinity.

Conclusions

RSV is an important viral pathogen that causes significant morbidity, utilization of health care resources, and impact on secondary disease processes. While there are significant biological and historical barriers that have delayed vaccine development, the current technologies available to understand the fundamental basis of RSV-induced disease and immunity and to develop new vaccine approaches should make this possible. Unlike other difficult vaccine targets like HIV, HSV, and HCV, there is a known immune correlate of protection, which is neutralizing antibody to the F glycoprotein. This provides a foundation on which to build a safe and efficacious vaccine to reduce the disease burden caused by RSV.

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Table 1

Major Challenges for RSV Vaccine Development

Early age of infection
Evasion of innate immunity
Failure of natural infection to induce immunity that prevents reinfection
Legacy of vaccine-enhanced illness
Failure of animal models to fully recapitulate pathogenesis of human RSV

Table 2

Principles of RSV Vaccine Development

Safety and Immunogenicity
• Induce neutralizing antibody for efficacy and manage T cells for safety
• Induce CD8 T cells and avoid Th2 responses
Timing
• Immunize early enough to protect majority of infants
• Ideally, vaccine will be first RSV antigenic exposure in order to establish an immunological paradigm different than natural infection
• Immunize late enough to avoid most maternal antibody, establish immunological maturity, and improve safety
Manufacturing and delivery
• Robust manufacturing
• Genetic and storage stability
• Parenteral delivery to avoid association with adverse airway events (apnea), although mucosal delivery would be optimal for local immunity

Table 3

Major Research & Development Opportunities to Facilitate RSV Vaccine Development

Clinical and Epidemiology
• Baseline prevalence of apnea and other rare events in neonates
• Incidence and illness severity data from more international sites
• Development of additional clinical trial sites in both northern and southern hemispheres
• Better diagnostic tools for endpoint measurements in clinical vaccine studies
Virology
• Better understanding of antigenic diversity and its role in year-to-year epidemic severity
• Production of more antibody reagents for both basic and clinical studies
• Structural data at level of whole virus and individual proteins
• Better chemical definition of viral glycoproteins
• Identification of the cellular receptor(s) for RSV
Immunology
• Biological mechanisms for immune modulation and failure of protective immunity
• Immunological features of the RSV-infected neonate (0-4 months of age)
• Definition of more B and T cell epitopes
• Better assays for immune response measurements in humans
Pathogenesis
• Biological mechanisms of apnea and potential role of RSV infection
• Immune, developmental, mechanical, structural basis for asthma/airway hypersensitivity and impact of RSV infection