Human respiratory syncytial virus (RSV) is a highly infectious, ubiquitous virus that is the leading cause of serious respiratory tract disease in children under one year of age worldwide (for reviews, see Brandenburg et al., 2001; Collins et al., 1999, 2001; Crowe et al., 1997; Dudas and Karron, 1998). RSV accounts for nearly one-quarter of hospitalizations due to pediatric respiratory tract disease, and in the United States accounts for 85,000 to 144,000 hospitalizations annually in infants of less than one year of age (Shay et al., 1999). RSV reinfection is gaining recognition as an important cause of disease in adults, especially the elderly, and in immunocompromised individuals. An RSV vaccine is not yet available. The primary need is for the pediatric population, with the second major target population being the elderly, for which RSV has an impact approaching that of nonpandemic influenza virus. Most of the candidate vaccines described below involve expression from cDNA, which is called reverse genetics (Berg, 1993).

RSV is an enveloped virus of the Paramyxovirus family (order Mononegavirales). Its genome is a single negative-sense RNA of 15.2 kilobases that is transcribed into 10 mRNAs encoding 11 proteins (the M2 mRNA encodes two proteins, M2-1 and M2-2 from overlapping open reading frames (ORFs)) (Fig. 1). N, P, L, and M2-1 are the nucleocapsid/polymerase proteins; M2-2 appears to play a regulatory role in RNA synthesis; M is a matrix protein; NS1 and NS2 are nonstructural proteins recently identified as antagonists of the interferon-α/β-mediated antiviral state (Schlender et al., 2000); and SH, G, and F are transmembrane glycoproteins. G and F are the only antigens that induce neutralizing antibodies and are the major protective antigens. Infectious recombinant RSV can be produced from transfected plasmids expressing a positive-sense copy of viral antigenomic RNA together with the nucleocapsid/polymerase proteins (Collins et al., 1995; Jin et al., 1998).

A number of characteristics of RSV biology and epidemiology complicate vaccine development. RSV does not replicate efficiently in vitro, and careful handling is necessary to maintain infectivity. RSV exists in two antigenic subgroups (A and B) that differ most prominently in the G protein, and both subgroups would be desirable in an RSV vaccine. Reinfection by RSV is common, although disease is reduced: this indicates that natural immunity is incomplete and poses a challenge to vaccination. Because the peak incidence of serious RSV disease is at 2–7 months of age, a pediatric RSV vaccine should be given shortly after birth. However, immune responses in the very young infant are reduced due to immunological immaturity (Delespesse et al., 1998; Murphy et al., 1986a) and the immunosuppressive effects of maternally derived RSV-specific serum IgG typically present in this age group (Crowe et al., 2001).

The chimpanzee is the only experimental animal that approaches the human in permissiveness to RSV replication and disease (Brandenburg et al., 2001; Belshe et al., 1977; Collins et al., 1990; Whitehead et al., 1998) but is limited in availability. Convenient experimental animals such as mice and cotton rats are nonnatural hosts that are semipermissive for RSV replication and do not exhibit authentic RSV disease. An accurate evaluation of the safety and efficacy of a pediatric RSV vaccine can only be made in clinical studies involving the target population, namely RSV-naïve 1- to 2-month-old infants possessing maternally derived antibodies (Wright et al., 2000).

Vaccine-associated disease potentiation and RSV protein vaccines. A Formalin-inactivated RSV (FI-RSV) vaccine that was evaluated in infants and children in the 1960s was found to be poorly protective and, unexpectedly, was associated with an increased frequency and severity of RSV disease upon subsequent natural infec-
also was shown to be a poor inducer of CD8 upon subsequent infection (Connors et al., 2001). The M2 mRNA contains two overlapping ORFs, M2-1 and M2-2, shown as small rectangles within the M2 box. The thin horizontal lines at the left- and right-hand ends represent the 3'-leader and 5'-trailer extragenic regions. Examples of viable gene-deletion mutants are shown with the deleted gene(s) or ORF indicated in black (Bermingham and Collins, 1999; Jin et al., 2000; Teng et al., 2001). The relative efficiency of replication in cell culture and in the respiratory tract of seronegative chimpanzees is scored from 0 to 5+. nd: not done. The viruses are arranged from the top in the order of increasing attenuation in chimpanzees or rodents.

FIG. 1. Diagrams illustrating the RSV gene map (negative-sense RNA, 3' to 5') and examples of gene deletions that result in viable, attenuated vaccine candidates. The 10 RSV genes are shown as boxes; each encodes a single mRNA and is flanked by short gene-start and gene-end signals that guide sequential transcription. The genes are separated by short intergenic regions except for the M2 and L genes, which overlap (Collins et al., 2001). The M2 mRNA contains two overlapping ORFs, M2-1 and M2-2, shown as small rectangles within the M2 box. The thin horizontal lines at the left- and right-hand ends represent the 3'-leader and 5'-trailer extragenic regions. Examples of viable gene-deletion mutants are shown with the deleted gene(s) or ORF indicated in black (Bermingham and Collins, 1999; Jin et al., 2000; Teng et al., 2001). The relative efficiency of replication in cell culture and in the respiratory tract of seronegative chimpanzees is scored from 0 to 5+. nd: not done. The viruses are arranged from the top in the order of increasing attenuation in chimpanzees or rodents.

The idea that the immune response to RSV antigens could increase RSV disease was unexpected, since natural RSV infection induces an immune response that is protective, albeit incompletely, and reduces rather than enhances disease associated with reinfection. Retrospective studies showed that FI-RSV induced serum antibodies that bound RSV antigen but did not efficiently neutralize the virus (Murphy et al., 1986b), a finding that has been confirmed in experimental animals and likely explains the poor protective efficacy. The original FI-RSV vaccinees also exhibited a heightened RSV-specific lymphoproliferative response (Kim et al., 1976). This is consistent with more recent studies in mice and cotton rats, where disease potentiation by FI-RSV was shown to depend on an increased stimulation of Th2 CD4+ T lymphocytes (Openshaw et al., 2001; Connors et al., 1994; Graham et al., 1993). This mediating enhanced disease is not yet clear. In contrast, passive transfer of serum antibodies from FI-RSV-immunized animals to naive animals did not enhance disease upon subsequent infection (Connors et al., 1992). FI-RSV also was shown to be a poor inducer of CD8+ T cells, which is not unusual for a protein vaccine. This might account for the heightened Th2 response to FI-RSV, since recent studies in mice showed that CD8+ T cells down-regulate Th2 cells during the immune response to RSV antigens (Hussell et al., 1997; Srikiatkhachorn and Braciale, 1997b). By this model, vaccines that do not efficiently induce CD8+ T cells, such as soluble antigens like FI-RSV, would have the propensity to prime for enhanced disease. In contrast, the strong CD8+ T cell response characteristic of natural RSV infection could account for the absence of disease potentiation. It also has been suggested that the G protein has unusual antigenic properties that favor an atypical inflammatory response (Hancock et al., 1996).

Disease potentiation in the rodent models also has been observed with experimental vaccines consisting of purified RSV F and G protein, and thus indeed might be a general concern with RSV protein vaccines (Graham et al., 1993; Connors et al., 1992; Hancock et al., 1996; Murphy et al., 1990). This might be improved by use of an adjuvant that alters the T lymphocyte response (Prince et al., 2001), although none are presently approved for human use. Unfortunately, it may be difficult to convincingly establish that immunization with a candidate pediatric protein vaccine does not potentiate RSV disease since studies in experimental animals are of limited predictive value for the human, and clinical studies in RSV-naive infants likely would pose unacceptable risk. However, disease potentiation is not observed if FI-RSV is administered as a boost to animals that have already been infected with RSV (Graham et al., 1993; Waris et al., 1997). Thus, while an RSV protein vaccine might not be appropriate as the first immunization for the young infant, it should be suitable to boost immunity in individuals who have already experienced one or more natural infections. Recipients of such a protein vaccine could include the elderly as well as individuals at high risk for RSV disease such as due to congenital heart disease or chronic lung disease.

Clinical trials have been reported for two RSV protein vaccines, all involving vaccinees of 24 months or older. The first is PFP, which consists of full-length F protein purified from RSV-infected cells (Hancock et al., 1995). PFP has been shown to be safe and moderately immunogenic in, for example, children with chronic lung disease (Groothuis et al., 1998) and the elderly (Falsey and Walsh, 1997). The second protein vaccine is BBG2Na, a
immunogenic in adults (Power et al., 2001a). BBG2Na was safe and moderately immunogenic in adults (Power et al., 2000b). Because the BBG2Na vaccine is produced in bacteria, its G moiety is devoid of glycosylation, and evidence has been presented that this may somehow preclude disease potentiation (Power et al., 2001a, 2001c). Other experimental RSV protein vaccines exist, including a baculovirus-expressed chimeric protein created by fusing the F and G ORFs (Kakuk et al., 1993), as well as synthetic peptides; these other protein vaccines are less far along in development and would follow the same principles as PFP and BBG2Na.

**Live-attenuated RSV vaccines.** A live-attenuated RSV vaccine administered intranasally would mimic natural infection without causing disease. The lack of disease potentiation associated with RSV infection is a critical safety advantage of the live vaccine strategy (Wright et al., 2000; Waris et al., 1997). The mucosal route of immunization also has the advantage of directly stimulating local immunity and of reducing the immunosuppressive effects of maternally derived antibodies (Crowe et al., 1995). It is anticipated that a live RSV vaccine would be administered two or three times during the first year of life to boost immunity. A live RSV vaccine also could be used as a vaccine for older seropositive individuals but would have to be less attenuated than one for pediatric use (Gonzalez et al., 2000).

Development of a live-attenuated RSV vaccine began in the 1960s using conventional methods such as extensive passage in vitro or chemical mutagenesis (Crowe et al., 1997; Dudas and Karron, 1998). The most promising mutants were made by subjecting wild-type RSV (strain A2, subgroup A) to extensive cold-passage in vitro, resulting in a moderately attenuated mutant called cp-RSV that was then subjected to two rounds of chemical mutagenesis to yield temperature-sensitive (ts) derivatives. Several promising mutants, cpts-248/955, cpts-530/1030, cpts-530/1009, and cpts-248/404, were evaluated in seropositive children and older seronegative children, and cpts-248/404 was deemed to be sufficiently attenuated to be evaluated in 1- to 2-month-old RSV-naive infants, the target vaccine age group (Wright et al., 2000). In this age group, more than 80% of vaccinees were infected by cpts-248/404, shed a moderate amount of virus, had a significant rise in RSV-specific serum IgA antibodies, and were highly resistant to reinfection with a second vaccine dose given 1 month later. The antibody response to the G protein was more frequent and of greater magnitude than that to the F protein. However, a majority of vaccinees experienced brief nasal congestion following the initial immunization, indicating that the virus should be further attenuated. Also, a single individual shed a low, sporadic amount of virus with partial loss of the ts and attenuation phenotypes, indicating that a greater level of phenotypic stability would be desirable. Importantly, these studies provide evidence that effective immunization of young infants with a live RSV vaccine is feasible.

The strategy shifted to employ reverse genetics to systematically introduce attenuating mutations into infectious recombinant RSV strain A2. The cp-RSV and cpts-RSV mutants described above were sequenced in their entirety. Identified mutations were evaluated phenotypically by introduction into the wild-type recombinant virus (Collins et al., 1999). The cp-RSV mutant had five amino acid substitutions in the N, F, and L proteins, and each of the cpts-RSV mutants mentioned above had in addition a unique set of two ts attenuating point mutations. These involved various amino acid substitutions in the L polymerase protein as well as a single noncoding nucleotide change in the transcription gene-start signal of the M2 gene. Because RSV does not grow robustly in vitro, preferable attenuating mutations are ones that restrict replication in vivo but not in vitro, permitting efficient vaccine production. ts mutations fulfill this requirement because growth in vitro can be performed at reduced, permissive temperature. ts mutations also have the advantage of preferentially restricting replication in the warmer lower respiratory tract, reducing the risk of serious disease.

The deletion of nonessential genes provides another method of attenuation, one that should be very stable. Remarkably, five RSV genes, namely NS1, NS2, SH, G, and M2-2, can be deleted or silenced individually and in certain combinations without much effect on virus yield in vitro (Fig. 1) (Bermingham and Collins, 1999; Jin et al., 2000; Karron et al., 1997; Teng et al., 2001). Deletion of the G gene would be unlikely to be useful since G was the antigen that elicited the greatest and most frequent response in infants and since virus lacking G appears was over-attenuated in vivo (Karron et al., 1997; Teng et al., 2001). The other deletion mutants exhibited a range of attenuation in chimpanzees. In comparison to cpts-248/404, they could be ranked in order of increasing attenuation as follows: ΔSH < ΔNS2 < cpts-248/404 < ΔNS1 < ΔM2-2 (Teng et al., 2001). Thus, these gene deletions represent promising attenuating mutations for vaccine purposes, either on their own or in combination with point mutations described previously. A virus such as ΔNS2 that is less attenuated than cpts-248/404 might be a vaccine candidate for seropositive individuals in whom cpts-248/404 is over-attenuated (Gonzalez et al., 2000).

Another method of attenuation is based on bovine RSV (BRSV), a closely related animal counterpart of human RSV that is attenuated in primates due to a natural host range restriction (Buchholz et al., 2000). BRSV itself is over-attenuated and insufficiently immunogenic in sero-
negative chimpanzees (Buchholz et al., 2000). Growth was improved by replacing the G and F genes of BRSV with their human RSV counterparts, although the resulting chimeric virus remained over-attenuated (Buchholz et al., 2000). The systematic replacement of additional BRSV genes with their human RSV counterparts should yield viruses with a range of growth properties that can be evaluated as vaccines. Host range recombinants, such as deletion mutants, would be anticipated to be phenotypically stable, as discussed elsewhere (Buchholz et al., 2000; Schmidt et al., 2000).

Thus, four types of attenuating mutations are available for insertion in various combinations into recombinant RSV: the non-ts cp-RSV point mutations; the ts point mutations in the L protein and M2-1 gene-start signal identified in the cpts-RSV mutants; the gene deletions; and host-range-restrictive BRSV genes. The cp and ts amino acid point mutations in the biologically derived viruses each involved a single nucleotide substitution, and in many cases it was possible to introduce the attenuating amino acid assignment into recombinant virus using two nucleotide changes relative to wild-type. This should greatly reduce the frequency of reversion to the wild-type assignment, although it does not preclude mutation by single nucleotide substitution to another assignment with possible reduction in attenuation.

Figure 2 shows an example of a recombinant vaccine candidate, designated rA2cp248/404/1030ΔSH, that is a recombinant version of cpts-248/404 that has been further attenuated by the inclusion of an additional ts mutation, called 1030, from cpts-530/1030 as well as deletion of the SH gene. This virus was more ts and attenuated and resulted in a lower level of virus shedding in seronegative children compared to cpts-248/404 and represents a promising vaccine candidate (R. Karron, unpublished data). This illustrates how attenuating mutations from various viruses can be combined in desired combinations to fine-tune the level of attenuation. Our experience is that attenuation can be fine-tuned in a reasonably systematic way, although the level of attenuation specified by a combination of attenuating mutations is not always the sum of the individual mutations, and some trial and error is involved. A few combinations of point mutations in L or of gene deletions have not yielded viable virus (Jin et al., 2000; Whitehead et al., 1999a). A recombinant vaccine virus constructed using identified mutations would thus have a defined basis of attenuation that can be readily monitored during production and use, and cDNA provides a stable vaccine seed.

Additional means of attenuation of recombinant RSV can be devised. To date, a number of attenuating mutations have been identified by trial and error, including point mutations in the L protein, a point mutation in the leader region, and C-terminal deletions of the M2-1 protein (Tang et al., 2001). Based on studies with vesicular stomatitis virus (VSV), attenuation can be achieved by changing the gene order (Wertz et al., 1998). This strategy could be applied to RSV provided that it does not reduce the efficiency of growth in vitro, which otherwise could interfere with vaccine production. An attenuating mutation identified in one mononegavirus sometimes can be "transferred" to another, particularly if it involves a conserved residue. For example, an attenuating point mutation in the Sendai virus C protein was transferred to the corresponding position in the C protein of recombinant human parainfluenza virus type 3 (HPIV3), resulting in attenuation of the HPIV3 recipient (Durbin et al., 1999).

Reverse genetics was used to expedite development of an RSV subgroup B vaccine, which faced the obstacles of a lack both of promising vaccine candidates and of a recombinant recovery system. Replacement of the G and F genes of recombinant RSV A2 (subgroup A) with their counterparts of strain B1 of subgroup B produced a wild-type-like chimeric AB virus bearing the protective antigens of subgroup B (Whitehead et al., 1999b). Attenuated AB viruses were readily produced using attenuated versions of the A2 backbone and are in preparation for clinical evaluation. Alternatively, the subgroup B G gene, representing the more divergent protective antigen, can be expressed as an added gene from a recombinant subgroup A virus (Jin et al., 1998).

Reverse genetics was used to increase the efficiency of protective antigen expression by moving the G and F genes from their natural positions as the seventh and eighth genes in the gene order to promoter-proximal positions, resulting in an increase in their expression (C. Krempl, B. R. Murphy, and P. L. Collins, unpublished results). It also will be of interest to investigate whether modifying the codon usage of the F and G mRNAs to employ alternative codons associated with increased translational efficiency can promote more efficient expression of these protective antigens than the wild-type virus. Interestingly, silencing the M2-2 ORF resulted in a virus in which transcription and antigen expression was increased, whereas RNA replication and virus production were decreased. This suggests that, in a wild-type infection, the M2-2 protein down-regulates transcription and up-regulates RNA replication. Ablation of this protective regulatory function provides a novel phenotype that might increase vaccine immunogenicity.

In principle, a recombinant RSV vaccine virus could be
used as a vector for additional genes expressing protective antigens for heterologous pathogens such as HPIV3, but in practice the addition of large genes can drastically reduce the growth efficiency of RSV. However, small genes are tolerated, and a number of recombinant RSVs expressing immunomodulatory proteins such as cytokines and chemokines are being evaluated as a means of modifying and increasing the immune response to an attenuated virus. For example, expression of granulocyte-macrophage colony-stimulating factor (GM-CSF) by recombinant RSV following intranasal inoculation of mice resulted in a dramatic increase in the number of pulmonary dendritic cells and macrophages, as potential antigen presenting cells (Bukreyev et al., 2001).

It also might be possible to alter recombinant RSV to obtain improved characteristics of immunogenicity and reduced pathogenesis. For example, the RSV G protein was recently shown to be a structural and functional mimic of fractalkine (Tripp et al., 2001), a proinflammatory CX3C chemokine that mediates leukocyte migration and adhesion. It is not yet known whether this mimicry interferes with the host immune response or is a factor in pathogenesis during RSV infection, but if so this activity could be ablated by mutating the CX3C motif in G since this does not affect infectivity or virus growth in vitro and in mice (M. N. Teng and P. L. Collins, unpublished observations). As a second example, the G protein is expressed in part as a secreted form that arises from translational initiation at the second methionyl codon. The secreted form appears to prime for an atypical pulmonary inflammatory response in mice (Johnson et al., 1998). Therefore, it might be desirable to ablate its expression by mutation, which can be done without reducing infectivity or growth (Teng et al., 2001). It also will be interesting to determine whether deletion of the NS1 and NS2 genes, which encode antagonists of the interferon-α/β-mediated antiviral state, affects the immune response in humans.

**Vectored RSV vaccines.** This strategy is to express the G and F neutralization antigens in vivo from an inoculated recombinant vector. This approach was evaluated initially with recombinant vaccinia viruses expressing the RSV F or G protein. These recombinants were highly immunogenic and protective in mice and cotton rats, but when administered parenterally to chimpanzees they induced low to moderate levels of RSV-neutralizing antibodies and conferred inconsistent protection (Crowe et al., 1997). Replication-competent adenovirus expressing the F protein also was immunogenic and protective in rodents and dogs, but did not induce significant resistance to RSV infection in a chimpanzee (Crowe et al., 1997).

Salmonella and staphylococcus have been investigated as possible live recombinant vectors for RSV antigens, although development is at an early phase (Cano et al., 2000). DNA vaccines have been shown to be immunogenic and protective in mice (Li et al., 2000). However, DNA vaccines typically are not highly immunogenic on their own in primates and humans, usually are given parenterally rather than topically, are readily suppressed by existing antibodies, and require one or more boosts that would be difficult to administer in the short time window between birth and the peak of serious RSV disease.

Heterologous mononegaviruses have been explored as vectors for RSV antigens. One promising example is based on a chimera between HPIV3 and its antigenically related bovine counterpart, BPIV3. BPIV3 is attenuated in primates due to a natural host range restriction. It has been evaluated as a vaccine against HPIV3 and was found to be immunogenic, safe, and genetically stable in seronegative infants and children (Karron et al., 1996). Since HPIV3 and BPIV3 have antigenic differences, the bovine virus was next modified by reverse genetics so that its major protective antigen genes F and HN (the functional counterparts of RSV F and G) were replaced with F and HN of HPIV3. This resulted in the chimeric virus rB/HPIV3, a vaccine candidate for HPIV3 that combines the major protective antigens of HPIV3 with the attenuated backbone of BPIV3 (Schmidt et al., 2000) (Fig. 3). Next, the RSV G and F glycoproteins were inserted singly or together into rB/HPIV3, with a promoter-proximal insertion site chosen to maximize antigen expression (Schmidt et al., 2002). Recombinants were made representing both RSV antigenic subgroups (Fig. 3). The chimeric rb/HPIV3-RSV viruses replicated efficiently in vitro and expressed high levels of the RSV G and F glycoproteins. For example, the rb/HPIV3-G chimera expressed several-fold more G protein than either the RSV or the vaccinia-G recombinant mentioned above. When inoculated into the respiratory tract of rhesus monkeys, the rb/HPIV3-RSV chimeras were somewhat more attenuated than their rB/HPIV3 parent, presumably due to the...
presence of the insert(s), but nonetheless were highly immunogenic against both RSV and HPIV3. As another example, recombinant VSVs were constructed that express RSV G or F, either as added genes or in place of the single VSV glycoprotein G, but were poorly immunogenic in mice (Kahn et al., 2001). This suggests that the biological characteristics of the vector virus, rather than efficient expression alone, are important determinants of immunogenicity and protective efficacy.

The rB/HPIV3-RSV-A and -B chimeric viruses provide a trivalent pediatric vaccine against RSV-A, RSV-B, and HPIV3. HPIV3 is second only to RSV in importance as a pediatric viral respiratory tract pathogen. Thus, the vector itself is a needed vaccine rather than simply a carrier. RSV and HPIV3 cause disease in approximately the same age group, and hence it is desirable to have a combined RSV/HPIV3 vaccine. Similar to RSV, PIV3 is pneumotropic and thus is well suited as a vector for intranasal immunization. PIV3 should be an extremely safe vector since it does not cause disease outside of the respiratory tract, and since the nonpediatric population has immunity to HPIV3. There is considerable experience in administering PIV3 to children and infants. These advantages do not hold for most other potential vectors for RSV antigens. Interestingly, the rB/HPIV3-RSV chimeric viruses were not neutralized by RSV-specific antibodies, raising the possibility that a PIV-vectored RSV vaccine might be particularly useful as a booster immunization in infants previously immunized with an RSV vaccine.

In conclusion, a number of promising live-attenuated RSV strains produced by reverse genetics are undergoing clinical evaluation as candidate vaccines for young infants. Two promising protein vaccines, one of which is of recombinant origin, are being evaluated as booster vaccines for RSV-experienced children and adults. A less-attenuated live RSV vaccine also could be used in these groups. Multivalent live vectored vaccines might simplify and expedite vaccine development and administration. The development of safe and effective RSV vaccines would benefit from a more complete understanding of RSV immunobiology, in particular, why immunity to RSV is less complete and durable compared to that, for example, of influenza virus or rhinovirus.

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