Chimeric Subgroup A Respiratory Syncytial Virus with the Glycoproteins Substituted by Those of Subgroup B and RSV without the M2-2 Gene Are Attenuated in African Green Monkeys

Xing Cheng, Helen Zhou, Roderick S. Tang, Mary G. Munoz, and Hong Jin

Aviron, 297 N. Bernardo Avenue, Mountain View, California 94043

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INTRODUCTION

Respiratory syncytial virus (RSV) is the leading cause of serious viral respiratory infection in infants and children worldwide. Despite decades of investigation, no safe and effective vaccines are available to prevent diseases caused by RSV infection. A number of live attenuated RSV candidate vaccines, generated by cold passage and/or chemical mutagenesis, have been evaluated in animals and humans (Crowe et al., 1969, 1974; Gharpure et al., 1969; Hsu et al., 1971; Richardson et al., 1977). These previous vaccine candidates have been inadequately attenuated and in some circumstances genetically unstable, rendering them unsafe for young children (Hodes et al., 1974; Kim et al., 1973; Wright et al., 1976, 2000). Recently, using the reverse genetics system developed for RSV (Collins et al., 1995; Jin et al., 1998), a large panel of cDNA-derived attenuated RSV have been obtained (reviewed by Collins et al., 1999). A number of these attenuated RSV strains are currently being evaluated for use as vaccines.

RSV is an enveloped virus and contains a single-stranded, negative-sense RNA genome of 15,222 nucleotides (nt). Ten subgenomic mRNAs are encoded by the viral genome and are translated into 11 proteins: the nucleoprotein (N), the phosphoprotein (P), the major polymerase protein (L), the matrix protein (M), the glycoprotein (G), the fusion protein (F), two nonstructural proteins (NS1 and NS2), the small hydrophobic protein (SH), and the M2-1 and M2-2 proteins. The G and F proteins are the major RSV surface antigens that elicit neutralizing antibodies in vivo. Two antigenically diverse RSV subgroups (A and B) have been distinguished on the basis of antigenic and sequence divergence. Within either subgroup, the G and F proteins exhibit high degrees of antigenic similarity. However, between subgroups, extensive differences are observed for the G protein. The antigenic diversity for the G protein between the two subgroups can be as great as 95% (Johnson et al., 1987b). The F glycoprotein is relatively more conserved between the two subgroups. Although there is 91% identity between the amino acid sequences of the subgroup A and B F protein, the antigenic diversity can differ by as much as 50% (Johnson et al., 1987a). This antigenic diversity enables both subgroups to circulate in a community at the same time (Reviewed by McIntosh and Chanock, 1990; Sullender, 2000). Although infection with subgroup A or B RSV in experimental animals induces a high level of resistance against replication of homologous or heterologous sub-
group RSV, infection with attenuated RSV induced better protection against homotypic virus than heterotypic RSV (Crowe et al., 1997a). Recent studies of a RSV vaccine in young infants have shown that infants develop dominant immune response against the RSV G protein than the F protein (Wright et al., 2000). All these available data suggest that prevention of serious RSV diseases through vaccination would require bivalent vaccines containing antigenic components from both subgroups.

Previously, we described a reverse genetics system to generate recombinant RSV from cDNA (Jin et al., 1998) and have used this technology to attenuate subgroup A RSV (Jin et al., 2000a,b). To expedite the development of an attenuated subgroup B RSV vaccine, we used the infectious cDNA developed for the A2 strain to express heterologous subgroup B specific antigens. The construction of a chimeric RSV that expressed an additional G protein from a subgroup B virus in a recombinant A2 virus was described earlier (Jin et al., 1998). Here we describe a recombinant chimeric RSV, designated rA-GBFB, in which the G and F genes of subgroup A were replaced with those of subgroup B. This virus is designated rA-GBFB. In addition, the M2-2 gene was removed from rA-GBFB, designated rA-GFbΔM2-2, and this virus was shown to be more attenuated than rA-GFb.

African green monkeys (AGM) were evaluated as a nonhuman primate model for assessing the attenuation, immunogenicity, and protective efficacy of RSV vaccine candidates. We showed that rA2 replicated to high titers in both the upper and the lower respiratory tracts of AGM, whereas rA2ΔM2-2, rA-GFb, and rA-GFbΔM2-2 replicated poorly in the respiratory tracts of monkeys. However, they all induced sufficient immunity to protect animals from experimental challenge.

RESULTS

Construction of cDNA and recovery of RSV A/B chimeric virus

Previously, we constructed an infectious antigenomic cDNA encoding wt RSV strain A2 and its derivative bearing a deletion of the M2-2 gene. Here, these cDNAs were modified by replacing the G and F genes of the A2 strain with those of the subgroup B RSV 9320 strain to produce chimeric viruses expressing RSV subgroup B antigens. The gene-start and gene-end sequences are very conserved between the two RSV subgroups. Therefore, the complete G and F genes of 9320 including their own gene-start and gene-end signals were transferred to the A2 cDNA backbone (Fig. 1). The cDNA encoding the G and F genes of 9320 was obtained by RT/PCR and confirmed by sequence analysis. The constructed chimeric cDNA was designated pRSVA-GFb. pRSVA-GFbΔM2-2 was constructed by deleting the M2-2 gene from pRSVA-GFb. The M2 gene containing the deletion of the M2-2 open reading frame from rA2ΔM2-2 (Jin et al., 2000a) was introduced into pRSVA-GFb through the unique Mscl and BamHI restriction enzyme sites. Both chimeric viruses (rA-GFb and rA-GFbΔM2-2) were recovered from cDNA using the previously described rescue system (Jin et al., 1998). The recovered recombinant viruses were plaque-purified and amplified in Vero cells.

Characterization of the recombinant chimeric viruses in vitro

Expression of the subgroup specific proteins by the chimeric viruses was analyzed by Northern and Western blotting. Using strain-specific probes, 9320-specific G and F mRNAs were detected in cells infected with rA-GFb and rA-GFbΔM2-2 (Fig. 2A). The M2-2 gene was not deleted in cells infected with rA-GFbΔM2-2 (lane 5), confirming that the M2-2 gene was deleted from this chimeric virus. The 9320 strain-specific protein expression of the two chimeric viruses was also compared with that of rA2, rA2ΔM2-2, and wild-type 9320 (Fig. 2B). The F1 protein of rA-GFb and rA-GFbΔM2-2 showed the same rate of migration mobility as that of 9320; both migrated faster than that of A2. Western blotting analysis using strain-specific monoclonal antibodies confirmed that the G protein of subgroup B was expressed by rA-GFb and rA-GFbΔM2-2 (Fig. 2B). Immunoprecipitation using a polyclonal antibody specific to the M2-2 protein further confirmed the ablation of the M2-2 gene in rA2ΔM2-2 and rA-GFbΔM2-2. The M2-2 protein of RSV strain 9320 was not detected by the polyclonal antiserum raised against the M2-2 protein of strain A2 as there is only 62% homology between the M2-2 proteins of the two RSV subgroups.

Replication of chimeric viruses, rA-GFb and rA-
Replication of chimeric RSV in cotton rats

Cotton rats are susceptible to both subgroup A and subgroup B RSV infection. The levels of replication of rA-GF$_F$$delta$M2-2 and rA-GF$_F$ΔM2-2 in the nasal turbinates and lungs of cotton rats were compared with rA2, rA2ΔM2-2, and wild-type 9320 (Table 1). The replication of rA-GF$_F$ΔM2-2 was below the limit of detection by plaque assay in the nasal turbinates; its replication in lung tissue was reduced by about 3.6 log$_{10}$ compared to wild-type 9320 and by about 2.0 log$_{10}$ relative to rA2. The replication of rA2ΔM2-2 was not detected in the nasal turbinates and was 1.6 log lower in the lung compared to rA2. Removal of M2-2 from rA-GF$_F$ΔM2 further attenuated the chimeric virus. No virus replication was detected in either the nasal turbinates or the lungs of cotton rats infected with rA-GF$_F$ΔM2-2.

Although rA-GF$_F$ΔM2-2 were attenuated in cotton rats, both chimeric viruses induced sufficient immunity to protect the animals from homologous and heterologous RSV challenge (Table 1). rA-GF$_F$ΔM2-2 induced complete protection against subgroup B RSV challenge, but its protection against the heterotypic subgroup A RSV challenge was incomplete in cotton rats. A low level of A2 challenge virus replication was detected in the nasal turbinates of cotton rats previously infected with rA-GF$_F$ΔM2-2. The level of serum anti-RSV neutralizing antibody induced by rA-GF$_F$ΔM2-2 was 2.85-fold lower relative to that induced by wild-type 9320. Serum anti-RSV neutralizing antibody induced by rA-GF$_F$ΔM2-2 was approximately fourfold lower compared to that induced by 9320 and 1.5-fold lower than that of rA-GF$_F$. By comparison, the level of serum anti-RSV neutralizing antibody induced by rAΔM2-2 was similarly reduced by approximately twofold compared to that of rA2.

Replication of wt RSV and rA2ΔM2-2 in AGM

To investigate RSV attenuation and immunogenicity in primates, replication of recombinant RSV was further studied in AGM. Study A examined the replication of recombinant A2 and wild-type A2 virus in the respiratory tracts of AGM. RSV seronegative AGM were infected with 5.5 log$_{10}$ pfu of rA2 or wt A2 intranasally and intratracheally and virus shedding was monitored over a period of 12 days in both the upper and the lower respiratory tracts. As shown in Table 2, rA2 replicated well in both the upper and the lower respiratory tracts of AGM. rA2 reached a peak titer of 4.18 and 4.28 log$_{10}$ pfu/ml at each site, respectively, and shed virus over the same length of time as the wild-type A2 virus (Table 2, study A), though the peak titer of rA2 in the respiratory tracts of AGM was slightly lower than that obtained for wild-type A2 virus. Having confirmed a high level of replication of rA2 in AGM, rA2ΔM2-2 was evaluated for its attenuation, immunogenicity, and protective efficacy in AGM. In a separate study (study B, Table 2), rA2ΔM2-2 showed a
greatly reduced level of replication in both the nasopharynx and the trachea compared to rA2. Its peak titer in nasopharynx was reduced by 3.1 log₁₀, whereas the peak titer in the trachea was reduced by 3.25 log₁₀ compared to rA2. Despite the much lower level of replication in the respiratory tracts, rA2ΔM2-2 induced a significant level of serum anti-RSV neutralizing antibody. The antibody titer induced by rA2ΔM2-2 was about fourfold lower than that induced by rA2 at 3 weeks postinfection (Table 3). When challenged with wild-type A2 virus, rA2ΔM2-2 provided partial protection against wild-type RSV replication in the upper respiratory tract and much greater protection in the lower respiratory tract of immunized monkeys. Monkeys previously infected with rA2 were fully protected against wt A2 virus replication in both the upper and the lower respiratory tracts (Table 3). Although rA2ΔM2-2 did not provide complete protection in the respiratory tracts of immunized monkeys, it reduced virus shedding by 5 days. Two weeks after challenge, the level of serum anti-RSV neutralizing antibody from rA2ΔM2-2 infected monkeys approached that induced by rA2.

Replication of chimeric RSV and wild-type 9320 in AGM

We next compared the level of replication of chimeric rA-GaF₈ with that of wild-type 9320. RSV seronegative AGM were inoculated with 5.5 log₁₀ pfu of rA-GaF₈ or 9320 by intranasal and intratracheal instillation. The throat swab and tracheal lavage samples were collected over 12 days for virus quantitation. 9320 replicated to a level similar to that of wild-type A2 virus (Table 2). The peak titer of rA-GaF₈ at both sites of the respiratory tracts of the infected monkeys was about 1000-fold reduced compared to that of 9320. Animals infected with rA-GaF₈ shed virus for a shorter period of time than those infected with 9320. Despite its significantly attenuated replication, rA-GaF₈ provided complete protection when challenged with wild-type 9320. No challenge virus was detected in...
either the upper or the lower respiratory tracts of the monkeys previously immunized with rA-G_{F_0} (Table 3). Consistent with the level of protection seen in monkeys immunized with rA-G_{F_0}D_{M2-2}, the level of serum anti-RSV neutralizing antibody from these monkeys was similar to that observed for wild-type 9320-infected animals. rA-G_{F_0}D_{M2-2} was evaluated in AGM in a separate study (study C). The replication of rA-G_{F_0}D_{M2-2} was not detected in the upper respiratory tracts and a very low level of virus replication was detected in the lower respiratory tracts of the infected monkeys (Table 2). Since rA-G_{F_0}D_{M2-2} appeared to be more attenuated than rA-G_{F_0} and rA2-D_{M2-2}, an additional boosting dose was administered 4 weeks later. The boosting infection greatly augmented immune response and provided complete protection against wild-type 9320 RSV challenge. The level of serum anti-RSV neutralizing antibody induced by rA-G_{F_0}D_{M2-2} was about fourfold lower than that induced by rA-G_{F_0}. However, after a second dose of boosting infection, the level of serum neutralizing antibody was increased by about eightfold and it was further augmented by an additional twofold following subsequent wild-type RSV infections.

**DISCUSSION**

In an attempt to develop live attenuated RSV vaccine, we are using a recently developed reverse genetics system to attenuate RSV by introducing various mutations into the RSV genome. This approach has generated a number of attenuated subgroup A recombinant RSV by different groups (Jin et al., 2000a,b; Teng and Collins, 1999; Teng et al., 2000; Bermingham and Collins, 1999; Whitehead et al., 1999a,b). To expedite vaccine develop-

**TABLE 1**

<table>
<thead>
<tr>
<th>Virus*</th>
<th>Virus titer a (mean log_{10} pfu/g ± SE)</th>
<th>Neutralizing Ab titer (mean reciprocal log_{2})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NT</td>
<td>Lung</td>
</tr>
<tr>
<td>rA2</td>
<td>3.9 ± 0.13</td>
<td>3.57 ± 0.07</td>
</tr>
<tr>
<td>rA2D_{M2-2}</td>
<td>&lt;1.4</td>
<td>2.02 ± 0.12</td>
</tr>
<tr>
<td>Control</td>
<td>&lt;1.4</td>
<td>&lt;1.4</td>
</tr>
<tr>
<td>9320</td>
<td>2.8 ± 0.57</td>
<td>5.6 ± 0.05</td>
</tr>
<tr>
<td>rA-G_{F_0}</td>
<td>&lt;1.4</td>
<td>1.94 ± 0.31</td>
</tr>
<tr>
<td>rA-G_{F_0}D_{M2-2}</td>
<td>&lt;1.4</td>
<td>&lt;1.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Titer of challenge virus (mean log_{10} pfu/g ± SE) a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NT</td>
</tr>
<tr>
<td>A2</td>
<td>&lt;1.4</td>
</tr>
<tr>
<td>9320</td>
<td>2.8 ± 0.57</td>
</tr>
<tr>
<td>rA-G_{F_0}</td>
<td>&lt;1.4</td>
</tr>
<tr>
<td>rA-G_{F_0}D_{M2-2}</td>
<td>&lt;1.4</td>
</tr>
</tbody>
</table>

a Cotton rats were administered with 5.5 log_{10}PFU of virus intranasally under light anesthesia on day 0 and sacrificed on day 4.
b Virus titers from the nasal turbinates (NT) and lung tissues were determined by plaque assay.
c Serum RSV neutralizing antibody titers were determined by a complement-enhanced 50% plaque reduction assay with wt A2 or 9320.
d On day 21 of virus infection, cotton rats in groups of six were challenged with wt A2 or wt 9320 and the challenge virus titers from the nasal turbinates (NT) and lung tissues were determined by plaque assay.
e ND, not determined.

**TABLE 2**

<table>
<thead>
<tr>
<th>Virus*</th>
<th>AGM number</th>
<th>Virus shedding (days)</th>
<th>Virus peak titer (Mean log_{10} pfu ± SE) b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Nasopharyngeal swab</td>
</tr>
<tr>
<td>wt A2</td>
<td>4 (Study A)</td>
<td>8</td>
<td>4.67 ± 0.17</td>
</tr>
<tr>
<td>rA2</td>
<td>4 (Study A)</td>
<td>8</td>
<td>4.18 ± 0.18</td>
</tr>
<tr>
<td>rA2</td>
<td>4 (Study B)</td>
<td>9</td>
<td>3.44 ± 0.27</td>
</tr>
<tr>
<td>rA2D_{M2-2}</td>
<td>4 (Study B)</td>
<td>4</td>
<td>0.33 ± 0.26</td>
</tr>
<tr>
<td>9320</td>
<td>4 (Study B)</td>
<td>9</td>
<td>4.51 ± 0.18</td>
</tr>
<tr>
<td>rA-G_{F_0}</td>
<td>4 (Study B)</td>
<td>4</td>
<td>1.50 ± 0.42</td>
</tr>
<tr>
<td>rA-G_{F_0}D_{M2-2}</td>
<td>4 (Study C)</td>
<td>3</td>
<td>&lt;0.7</td>
</tr>
</tbody>
</table>

a African green monkeys were administered with 5.5 log_{10}PFU of virus intranasally and intratracheally. Nasopharyngeal swab samples were collected daily for 12 days, and tracheal-lavage samples were collected on days 3, 5, 7, and 10.
b Virus titers were determined in the nasopharyngeal swab and tracheal-lavage by plaque assay and only the peak titers are shown.
The recovered chimeric RSV (rA-G3F8) replicated efficiently in Vero cells, but its growth in HEp-2 cells was reduced by 5- to 10-fold relative to rA2. rA-G3F8 was attenuated in both the upper and the lower respiratory tracts of cotton rats. To determine whether the attenuation of rA-G3F8 was host specific, this chimeric virus was further evaluated in AGM that are genetically more closely related to humans than rodents. RSV infection in AGM is less well characterized and there is a wide range in the reported peak titer (Crowe et al., 1996b; Kakuk et al., 1993). Therefore, we first tested RSV infection in AGM using wild-type viruses. We showed that both subgroup A and subgroup B RSV replicated equally well in AGM and virus titers recovered from the upper and lower respiratory tracts of AGM approached those observed in infected chimpanzees (Crowe et al., 1994). When rA-G3F8 was evaluated in AGM, it showed a mean peak titer reduction of 3.0 log10 in the upper respiratory tract and a reduction of 2.59 log10 in the lower respiratory tract.

The level of attenuation of rA-G3F8 in AGM was consistent with what we observed in cotton rats. However, this result was somewhat different from that reported for a recently described chimeric RSV in which the G and F genes of A2 were replaced with those of RSV B1 strain (rAB1) (Whitehead et al., 1999b). Though rAB1 and rA-G3F8 are similarly attenuated in cotton rats, rAB1 was not attenuated in chimpanzees. In contrast to rA-G3F8, rAB1 replicated better than wt RSV B1 in both the upper and the lower respiratory tracts of chimpanzees (Whitehead et al., 1999b). Part of this discrepancy may be explained by the semipermissiveness of chimpanzees to wild-type subgroup B RSV infection. However, there exists the possibility that rA-G3F8 is more attenuated than rAB1 because of differences in the subgroup B strain surface antigens or constellation effects when these antigens are introduced into an A2 background. Chimerization of surface antigens resulting in an attenuated virus has been reported for several paramyxoviruses. A chimeric measles virus with the HN and F proteins replaced by the G protein of VSV was highly restricted in replication in vitro (Spielhofer et al., 1998). A chimeric Rinderpest virus in which the F and H proteins were replaced by the heterologous surface proteins of a closely related pestes-des-petits-ruminants virus was attenuated in vitro, as indicated by slow virus growth and low virus yield (Das et al., 2000). Most recently, it was reported that the PIIV3-
PIV2 chimeric virus, in which the F and HN genes of PIV3 were replaced by those of PIV2, was not attenuated in vitro, but it was highly attenuated in hamsters, AGM, and chimpanzees (Tao et al., 2000). On the other hand, the chimeric PIV3-PIV1 was not attenuated in vivo (Tao et al., 1998, 1999). Thus, it appears that chimerization of different heterologous proteins can result in different phenotypes. Though attenuated in AGM, rA-G_F_F_ induced significant levels of anti-RSV neutralizing antibody and provided complete protection against subsequent challenge with wild-type subgroup B RSV.

We previously reported that the recombinant A2 RSV lacking the M2-2 gene is attenuated in mice and cotton rats. In this study, we evaluated rA2ΔM2-2 for its attenuation, immunogenicity, and protection against wild-type RSV challenge in AGM. We showed that rA2ΔM2-2 was attenuated in the respiratory tracts of AGM and following challenge, much reduced replication of wild-type RSV was observed in animals previously infected with rA2ΔM2-2. The protection was higher in the lower respiratory tract than the upper respiratory tract. The level of replication and protection observed for rA2ΔM2-2 in AGM is very similar to that reported in a chimpanzee study for a similar recombinant RSV that had the M2-2 protein expression silenced (Birmingham and Collins, 1999; Teng et al., 2000). rA2ΔM2-2 may prove to be more attenuated in humans than a previously tested vaccine candidate cpts248/404 (Teng et al., 2000). cpts248/404 was neither sufficiently attenuated nor genetically stable in naive infants (Crowe et al., 1994; Wright et al., 2000). The serum anti-RSV neutralizing antibody titer induced by rA2ΔM2-2 was slightly lower than that induced by the wild-type RSV infection. However, the augmentation of neutralizing antibody titer after the challenge suggests that the immunogenicity of rA2ΔM2-2 could be enhanced by repeat administrations.

Since rA2ΔM2-2 exhibits many of the desired features in a live attenuated vaccine, we considered the deletion of the M2-2 gene an appropriate way to further attenuate the chimeric rA-G_F_F_. In vitro studies indicated that rA-G_F_F_2-2 had phenotypes similar to rA2ΔM2-2, exhibiting increased syncytial formation, reduced growth in HEp-2 cells, and unbalanced RNA transcription to replication. rA-G_F_F_2-2 was more attenuated than rA-G_F_ and rA2ΔM2-2 in both cotton rats and AGM. This attenuated replication in the host led to its reduced immunogenicity. Thus, although rA-G_F_F_ provided complete protection to both subgroup A and subgroup B RSV challenge, A-G_F_F_2-2 provided lower protection to subgroup A virus infection than to subgroup B strain infection in cotton rats. A very low level of serum anti-RSV neutralizing antibody was detected in monkeys infected with rA-G_F_F_2-2. The second dose administration of A-G_F_F_2-2 greatly augmented antibody response and provided complete protection against subsequent experimental challenge in AGM. Our data implied that a live attenuated RSV vaccine containing components from both subgroups need to be administered in multiple doses to achieve a higher level of durable immunity. rA-G_F_F_2-2, in combination with rA2ΔM2-2, may represent suitable vaccines for protecting against both subgroup A and subgroup B RSV infections.

MATERIALS AND METHODS

Cells and viruses

Monolayer cultures of HEp-2 and Vero cells (obtained from American Type Culture Collections, ATCC) were maintained in minimal essential medium (MEM) containing 5% fetal bovine serum (FBS). Wild-type RSV strains, A2 and 9320, were obtained from ATCC and grown in Vero cells. Modified vaccinia virus Ankara (MVA-T7) expressing bacteriophage T7 RNA polymerase was provided by Dr. Bernard Moss and grown in CEK cells.

Construction of chimeric cDNA clone

The wild-type RSV strain 9320, originally isolated in Massachusetts in 1977 and classified as subgroup B RSV (Hierholzer and Hirsch, 1979), was used in this study. The 9320 RSV was grown in Vero cells and the viral RNA was extracted from infected cell culture supernatant. A cDNA fragment containing the G and F genes of RSV 9320 was obtained by RT/PCR using the following primers: ATCAGGATCCAACATTGAATGGCAGC-AACC and CTGGCGATTGGAATCCGGTTTATGTAACATC-TGG (the BamHI sites engineered for cloning are in italics and 9320 specific sequences are underlined).

BamHI restriction enzyme sites were introduced upstream of the gene start sequence of G and downstream of the gene end sequence of F. The PCR product was first introduced into the TA cloning vector (Invitrogen) and the sequences were confirmed by DNA sequencing. The BamHI restriction fragment containing the G and F gene cassette of 9320 was then transferred into a RSV cDNA subclone pRSV(R/H) that contained RSV sequences from nt 4326 to nt 9721 through the introduced restriction sites engineered for cloning. A cDNA fragment containing the G and F genes of RSV 9320 was obtained by RT/PCR using the following primer pairs: BgII and BgII restriction enzyme sites engineered for cloning are in italics and 9320 specific sequences are underlined. BamHI restriction enzyme sites were introduced upstream of the gene start sequence of G and downstream of the gene end sequence of F. The PCR product was first introduced into the TA cloning vector (Invitrogen) and the sequences were confirmed by DNA sequencing. The BamHI restriction fragment containing the G and F gene cassette of 9320 was then transferred into a RSV cDNA subclone pRSV(R/H) that contained RSV sequences from nt 4326 to nt 9721 through the introduced restriction sites engineered for cloning. A cDNA fragment containing the G and F genes of 9320 was examined by sequencing analysis and then shuttled into the infectious RSV antigenic cDNA clone pRSV4G (Jin et al., 1998). The chimeric antigenic cDNA was designated pRSV-G_F_F_. To delete the M2-2 gene from pRSV-G_F_2, the Msc I (nt 7692) to BamHI (nt 8498) fragment from rA2ΔM2-2 which contained the
M2-2 deletion (Jin et al., 2000a) was introduced into pRSV-G<sub>F</sub><sub>B</sub>. The chimeric cDNA clone that lacks the M2-2 gene was designated pRSV-G<sub>F</sub><sub>B</sub>ΔM2-2.

Recovery of recombinant RSV

Recovery of recombinant RSV from cDNA was described previously (Jin et al., 1998). Briefly, HEp-2 cells in six-well plates at 80% confluence were infected with MVA at an m.o.i. of 5 pfu/cell for 1 h and then were transfected with full-length antigenomic plasmids (pRSV-G<sub>F</sub><sub>B</sub> or pRSV-G<sub>F</sub><sub>B</sub>ΔM2-2), together with plasmids expressing the RSV N, P, and L proteins using LipofectAMINE reagent (Life Technologies, Gaithersburg, MD). After incubating the transfected cells at 35°C for 3 days, the culture supernatants were passaged in Vero cells for 6 days to amplify rescued virus. The recovered recombinant viruses were biologically cloned by three successive plaque purifications and further amplified in Vero cells. Virus recovered from pRSV-G<sub>F</sub><sub>B</sub>-transfected cells was designated rA-G<sub>F</sub><sub>B</sub> and that from pRSV-G<sub>F</sub><sub>B</sub>ΔM2-2 transfected cells was designated rA-G<sub>F</sub><sub>B</sub>ΔM2-2. Virus titer was determined by plaque assay and plaques were enumerated by immunostaining using polyclonal anti-RSV A2 serum (Biogenesis, Sandown, NH).

Virus characterization

The expression of viral RNA for each recovered chimeric RSV was analyzed by Northern blotting. Total cellular RNA was extracted from virus-infected cells at 48 h postinfection. The RNA blot was hybridized with a γ<sup>32</sup>P-ATP-labeled oligonucleotide probe specific for the F gene of 9320 (GAGGTGAGGTACAATGCATTAATAGCAAGATGGAGGAAGA) or a γ<sup>32</sup>P-ATP-labeled probe specific for the F gene of A2 (CAGAAGC AAAACAAAATGTGACTGCAGTGAGGATTGTGGT). To detect the G mRNA of the chimeric viruses, RNA blots were hybridized with a 190-nt riboprobe specific to the G gene of 9320 or a monoclonal antibody against the G protein of strain 9320 or a monoclonal antibody against the G protein of A2 (Storch and Park, 1987).

Growth of chimeric RSV in vitro was compared with wild-type recombinant A2 (rA2) and rA2ΔM2-2. Growth-cycle analysis was performed in both HEp-2 and Vero cells. Cells grown in 6-cm dishes were infected with each virus at a m.o.i. of 0.01 or 0.1. After 1 h absorption at room temperature, the infected cell monolayers were washed 3 times with PBS and incubated at 35°C with 4 ml of Opti-MEM in an incubator containing 5% CO<sub>2</sub>. At various times postinfection, 200 μl of the culture supernatant was collected and stored at −70°C for virus titration. Each aliquot removed was replaced with an equal amount of fresh medium. Virus titer was determined by plaque assay in Vero cells on 12-well plates using an overlay of 1% methylcellulose and 1× L15 medium containing 2% FBS.

Virus replication in cotton rats

Virus replication in vivo was determined in respiratory pathogen-free Sigmodon hispidus cotton rats. Cotton rats in groups of 12 or 18 were inoculated intranasally under light methoxyflurane anesthesia with 10<sup>5.5</sup> pfu of virus per animal in a 0.1-ml inoculum. On day 4 postinoculation, six animals were sacrificed by CO<sub>2</sub> asphyxiation and their nasal turbinates and lungs were harvested separately. Tissues were homogenized and virus titers determined by plaque assay in Vero cells. Three weeks later, the remaining six animals were anesthetized, their serum samples were collected, and a challenge inoculation of 10<sup>5</sup> pfu of biologically derived wild-type RSV strain A2 or 9320 administered intranasally. To investigate the cross-protection of the chimeric viruses to heterologous RSV, six additional animals infected with rA-G<sub>F</sub><sub>B</sub> or rA-G<sub>F</sub><sub>B</sub>ΔM2-2 were also challenged with wt A2 RSV. Four days postchallenge, the animals were sacrificed and both nasal turbinates and lungs were harvested, homogenized, and virus titer determined by plaque assay. Serum neutralizing antibodies against RSV A2 or 9320 strain were determined by a 50% plaque reduction assay (Coates et al., 1966).

Virus replication in AGM

Recombinant RSV was evaluated for their replication, immunogenicity, and protective efficacy in AGM (Cercopithecus aethiops). AGM, obtained from St. Kitts with an average age of 4.2 years and body weight ranging from 2.2 to 4.3 kg, were used in the first study (study A) to compare the replication of rA2 with wild-type A2. The
were collected from rA-GBFB 4 weeks after boosting infection. NP and BAL samples were challenged with 5.5 log10 pfu of wt 9320 virus and 10 postinfection (Kakuk et al., 1993). On day 28 postinfection, serum samples were collected from each infected monkey and the monkeys were challenged with either wild-type A2 or 9320 at both the intranasal and the intratracheal sites with a dose of 10^3 pfu in a 1.0 ml inoculum. Replication of the challenge virus in the upper and lower respiratory tracts of monkeys was examined by quantitation of virus shed in NP and tracheal lavage specimens. The NP samples were collected daily for 10 days and BAL samples were collected on days 3, 5, 7, and 10 postinfection. Fourteen days after wild-type virus challenge, serum samples were collected for measurement of RSV neutralizing antibody. rA-GaFbΔM2-2 was evaluated in a separate study (study C). Four weeks after infection, a group of four monkeys were administered with an additional boosting dose of 5.5 log10 pfu of rA-GaFbΔM2-2 intranasally and intratracheally and monkeys were challenged with 5.5 log10 pfu of wt 9320 virus 4 weeks after boosting infection. NP and BAL samples were collected from rA-GaFbΔM2-2-infected monkeys the same as described for those infected with rA-GaFb. Serum samples were collected at day 0 (preserum), day 28 (preboosting), day 56 (postboosting), and day 70 (postchallenge). The levels of neutralizing antibody from monkeys infected with rA2 and rA2ΔM2-2 were determined by the plaque reduction assay using wild-type A2 virus. The levels of neutralizing antibody from monkeys infected with 9320, rA-GaFb, and rA-GaFbΔM2-2 were determined by a microneutralization assay (Cheng et al., manuscript in preparation). The virus shedding in the NP and BAL samples were quantitated by plaque assay using Vero cells.

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