Recombinant Respiratory Syncytial Virus That Does Not Express the NS1 or M2-2 Protein Is Highly Attenuated and Immunogenic in Chimpanzees

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Respiratory syncytial virus (RSV) is the leading etiologic agent of serious pediatric viral bronchiolitis and pneumonia worldwide and is responsible for approximately 100,000 hospitalizations and 4,500 deaths among infants and children in the United States per annum (7, 14, 25). In addition, RSV infection can cause severe respiratory illness in the elderly (23) and in immunocompromised individuals (28). To date, an effective licensed vaccine for RSV is not available despite the pressing need for such an agent.

Since 1967, our laboratory has focused on developing a live-attenuated RSV vaccine for intranasal administration. By mimicking a natural infection, such a vaccine should stimulate both cellular and humoral immunity and would obviate the potential disease that was observed with nonreplicating or subunit vaccines (7, 16, 24, 27). The intranasal route also partially abrogates the immunosuppressive effects of maternal antibodies present in the sera of young infants and stimulates both local and systemic immunity (10).

A number of live-attenuated RSV vaccine candidates have been developed by biological or recombinant methods and evaluated in animals and humans (8, 15, 16, 29, 30, 32). The most promising biologically derived candidate, a cold-passaged (cpts) virus called 248/404, was evaluated in RSV-naive 1- to 2-month-old infants and was found to be infectious, immunogenic, and protective against a second vaccine dose (33). However, some vaccinees experienced mild upper respiratory tract congestion, indicating that further attenuation is necessary. In addition, virus isolated late during the course of infection from a single vaccinee showed partial phenotypic reversion and loss of an attenuating mutation. Thus, our strategy to develop improved live-attenuated vaccine candidates has been (i) to use recombinant methods to combine attenuating mutations identified in a panel of biologically derived attenuated viruses including cpts248/404 and (ii) to develop new types of attenuating mutations by focusing on gene deletions which should be refractory to genetic reversion.

RSV is the prototype member of the Pneumovirus genus of the family Paramyxoviridae. Its genome is a single-stranded, negative-sense RNA of 15.2 kb that encodes 10 subgenomic mRNAs from which 11 proteins are translated. These proteins include the nucleocapsid N protein, phosphoprotein P, and large polymerase subunit L, which together comprise the minimal viral polymerase; fully processive transcription by the RSV polymerase requires the presence of the transcription antitermination factor M2-1 (6, 18, 19, 34). There are four envelope-associated proteins: the internal matrix (M) protein and three transmembrane surface proteins, namely, the attachment (G), fusion (F), and small hydrophobic (SH) proteins (7). Finally, RSV encodes two nonstructural proteins, NS1 and NS2, and also the M2-2 protein, whose status as structural or nonstructural is unknown. NS1 and M2-2 appear to have roles in RNA synthesis.

We previously described a reverse-genetics system for producing recombinant subgroup A RSV (rRSV) by coexpression of antigenic RNA and the N, P, L, and M2-1 proteins from cotransfected plasmids (5). One application of this system has been to identify viral genes that can be deleted or silenced without ablating replication in vitro but are still necessary for
The rA2ΔNS1 and rA2ΔM2-2 viruses were administered individually to juvenile RSV-seronegative chimpanzees by combined intranasal and intratracheal inoculation, as described previously (11). Since both viruses were attenuated in vitro, we chose to inoculate the animals with 10^3 PFU per ml per site, which is a 10-fold higher concentration than that typically used to inoculate chimpanzees. To monitor virus replication in the upper and lower respiratory tracts, respectively, nasopharyngeal swabs and tracheal lavage samples were collected at intervals over 10 days postinfection and subsequently were assayed for virus titer. The mean peak virus titer was determined for each group (Table 1). The chimpanzees were monitored daily for rhinorrhea, a symptom of upper respiratory tract illness, and the mean peak score was determined for each group (Table 1). Due to the limited availability of RSV-seronegative chimpanzees, the number of animals per group was small, making it necessary to include controls from previous studies in which we had evaluated biologically derived RSV strain A2 (wild-type RSV A2), rA2, rA2ASH, rA2ΔNS2, and a recombinant version of the above-mentioned cpts248/404 vaccine candidate (rA2cp248/404) (Table 1).

Levels of replication of rA2ΔNS1 and rA2ΔM2-2 were reduced more than 2,200-fold and more than 2,800-fold, respectively, in the upper respiratory tract compared to that of rA2 (Table 1). Shedding of rA2ΔNS1 or rA2ΔM2-2 was detected sporadically and at a low level beginning 2 to 7 days postinfection, and each animal shed virus over a period of 3 to 8 days (data not shown). Thus, the recovered virus was not carried over from the initial inoculum but represented replication near the level of detection over a period of several days. In the lower respiratory tract, the level of replication of rA2ΔNS1 was reduced more than 17,000-fold compared to that of rA2, while rA2ΔM2-2 was undetectable at all time points (greater than 55,000-fold reduction). It is important to note that the dose of rA2ΔNS1 and rA2ΔM2-2 used was 10-fold greater than that of rA2. Furthermore, both viruses were more attenuated than rA2cp248/404, which was given at the same dose, particularly in the case of rA2ΔM2-2, which was not recovered from the lungs of infected chimps. In addition, both rA2ΔNS1 and rA2ΔM2-2 were unusual in being equally restricted in the upper and lower respiratory tracts. In the upper respiratory tract, each virus was approximately 10-fold more restricted than cpts248/404 and 175-fold more restricted than rA2ΔNS2. Since upper respiratory tract congestion was observed during clinical evaluation of the cpts248/404 virus in 1- to 2-month-old infants (33) and since infants of that age are obligate nose breathers, mutations that confer a level of restriction of replication in the upper respiratory tract greater than that of cpts248/404 would be desirable for inclusion in a live-attenuated vaccine virus. Animals receiving rA2ΔNS1 or rA2ΔM2-2 had slightly more rhinorrhea than those infected with rA2cp248/404, though still less than that of animals infected with a 10-fold smaller dose of rA2. While it is possible that the absence of NS1 or M2-2 resulted in a virus that retained a moderate level of virulence but replicated poorly, we think that this possibility is unlikely. Our experience is that quantitation of rhinorrhea and the comparison of such values from different studies performed at different times can be somewhat subjective and hence not completely reproducible. We anticipate that further evaluation, including clinical studies, will show that the amount of residual virulence associated with rA2ΔNS1 and rA2ΔM2-2 will reflect their greatly reduced replication.

Despite the highly restricted replication of these viruses, immunization with either rA2ΔNS1 or rA2ΔM2-2 induced a level of RSV-neutralizing antibody in serum that was within threefold of that induced by rA2cp248/404 (Table 1). Further-
TABLE 1. rA2ΔNS1 and rA2ΔM2-2 are highly attenuated in both the upper and lower respiratory tracts of chimpanzees but are highly immunogenic

<table>
<thead>
<tr>
<th>Virus used to infect chimpanzees&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. of animals</th>
<th>Dose&lt;sup&gt;b&lt;/sup&gt; (per site, log&lt;sub&gt;10&lt;/sub&gt; PFU)</th>
<th>Mean peak virus titer&lt;sup&gt;c&lt;/sup&gt; (log&lt;sub&gt;10&lt;/sub&gt; PFU/ml) ± SE (Duncan grouping)</th>
<th>Mean peak rhinorrhea score&lt;sup&gt;d&lt;/sup&gt; (range, 0–4)</th>
<th>Mean neutralizing antibody titer in serum&lt;sup&gt;e&lt;/sup&gt; (reciprocal log&lt;sub&gt;2&lt;/sub&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type RSV A2&lt;sup&gt;f&lt;/sup&gt;</td>
<td>2</td>
<td>4.0</td>
<td>5.0 ± 0.35 (A)</td>
<td>3.0</td>
<td>&lt;3.3</td>
</tr>
<tr>
<td>rA2&lt;sup&gt;f&lt;/sup&gt;</td>
<td>2</td>
<td>4.0</td>
<td>4.9 ± 0.15 (A)</td>
<td>5.4 ± 0.05 (A)</td>
<td>&lt;3.3</td>
</tr>
<tr>
<td>rA2∆SH&lt;sup&gt;f&lt;/sup&gt;</td>
<td>3</td>
<td>4.0</td>
<td>4.6 ± 0.10 (A)</td>
<td>3.8 ± 0.31 (B)</td>
<td>&lt;3.3</td>
</tr>
<tr>
<td>rA2∆NS2&lt;sup&gt;f&lt;/sup&gt;</td>
<td>4</td>
<td>4.0</td>
<td>3.8 ± 0.41 (B)</td>
<td>1.4 ± 0.29 (C)</td>
<td>3.4</td>
</tr>
<tr>
<td>rA2cp248/404&lt;sup&gt;f&lt;/sup&gt;</td>
<td>4</td>
<td>5.0</td>
<td>2.5 ± 0.25 (C)</td>
<td>1.4 ± 0.37 (C)</td>
<td>3.4</td>
</tr>
<tr>
<td>rA2ΔNS1</td>
<td>4</td>
<td>5.0</td>
<td>1.6 ± 0.12 (D)</td>
<td>1.2 ± 0.43 (C)</td>
<td>&lt;3.3</td>
</tr>
<tr>
<td>rA2ΔM2-2</td>
<td>4</td>
<td>5.0</td>
<td>1.5 ± 0.09 (D)</td>
<td>&lt;0.7</td>
<td>1.8</td>
</tr>
</tbody>
</table>

<sup>a</sup> All recombinant-derived viruses contain the sites and HEK mutations (see the text), except for rA2ΔM2-2.

<sup>b</sup> Chimpanzees were inoculated by the intranasal and intratracheal routes with the indicated amount of virus in a 1-ml inoculum per site.

<sup>c</sup> Nasopharyngeal swab samples were collected daily for 10 days, and tracheal lavage samples were collected on days 2, 5, 6, 8, and 10. Mean peak titers were calculated and assigned to statistically similar groups by Duncan’s multiple-range test (α = 0.05). Means in each column with different letters are significantly different.

<sup>d</sup> The amount of rhinorrhea was estimated daily and assigned a score (0 to 4) that indicated extent and severity. Scores indicate severe (4), moderate (3), mild (2), trace (1), or no (0) rhinorrhea. Shown are the mean peak scores.

<sup>e</sup> Serum RSV-neutralizing antibody titers were determined by a complement-enhanced 60% plaque reduction assay using wild-type RSV A2 and HEp-2 cell monolayer cultures incubated at 37°C. RSV-seronegative chimpanzee serum used as a negative control had a neutralizing antibody titer of <3.3 log<sub>2</sub> reciprocal. Adult human serum used as a positive control had a neutralizing antibody titer of 11.4 log<sub>2</sub> reciprocal.

<sup>f</sup> Data from the study of Crowe et al. (10).

more, animals previously infected with either rA2ΔNS1 or rA2ΔM2-2 were highly resistant to the replication of wild-type RSV administered intranasally and intratracheally 56 days postimmunization (Table 2). The levels of protection in both cases were similar in the upper respiratory tract and somewhat lower in the lower respiratory tract than that seen with cpts248/404, both in mean peak titer and in mean days of shedding.

The challenge in developing a live-attenuated RSV vaccine is to eliminate residual virulence without compromising immunogenicity. Observations to date indicate that the severity of RSV disease is closely related to the level of RSV replication in the respiratory tract. It is possible that one or more attenuating mutations that reduce virulence through another mechanism will be identified; indeed, it was hoped that deletion of one or more of the nonessential RSV proteins, such as those described in the present paper, might reveal such a virulence factor. However, a factor of this nature has not yet been identified for RSV. Thus, the present method for attenuating RSV is to reduce its level of replication, which unfortunately can reduce its immunogenicity due to the reduced production of antigen. The attenuating mutations that we have identified to date include (i) a set of five amino acid substitutions in the N, F, and L proteins that were identified in cpts248/404, both in mean peak titer and in mean days of shedding.

TABLE 2. Infection of chimpanzees with rA2ΔNS1 or rA2ΔM2-2 induced significant protection against subsequent challenge with wild-type RSV A2 in the upper and lower respiratory tracts

<table>
<thead>
<tr>
<th>Immunizing virus</th>
<th>Inoculum dose&lt;sup&gt;a&lt;/sup&gt; (log&lt;sub&gt;10&lt;/sub&gt; PFU/ml)</th>
<th>No. of animals</th>
<th>Nasopharynx Mean no. of days of shedding ± SE</th>
<th>Mean peak titer ± SE</th>
<th>Trachea Mean no. of days of shedding ± SE</th>
<th>Mean peak titer ± SE</th>
<th>Mean peak rhinorrhea score</th>
</tr>
</thead>
<tbody>
<tr>
<td>rA2ΔNS1</td>
<td>5.0</td>
<td>4</td>
<td>2.8 ± 0.75</td>
<td>1.7 ± 0.46</td>
<td>1.0 ± 0.41</td>
<td>1.8 ± 0.73</td>
<td>1.0</td>
</tr>
<tr>
<td>rA2ΔM2-2</td>
<td>5.0</td>
<td>4</td>
<td>3.5 ± 0.87</td>
<td>2.3 ± 0.71</td>
<td>1.0 ± 0.71</td>
<td>1.7 ± 0.63</td>
<td>1.0</td>
</tr>
<tr>
<td>rA2ΔNS2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.0</td>
<td>4</td>
<td>ND</td>
<td>1.9 ± 0.30</td>
<td>ND</td>
<td>2.2 ± 0.77</td>
<td>1.0</td>
</tr>
<tr>
<td>cpts248/404&lt;sup&gt;e&lt;/sup&gt;</td>
<td>4.7</td>
<td>2</td>
<td>3.5 ± 0.50</td>
<td>2.3 ± 0.25</td>
<td>0</td>
<td>&lt;0.7</td>
<td>1.0</td>
</tr>
<tr>
<td>None&lt;sup&gt;e&lt;/sup&gt;</td>
<td>8.5 ± 0.50</td>
<td>2</td>
<td>5.0 ± 0.35</td>
<td>4.8 ± 0.30</td>
<td>6.0 ± 1.0</td>
<td></td>
<td>3.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Each virus was initially administered at the indicated dose in a 1.0-ml inoculum given intranasally and intratracheally.

<sup>b</sup> Chimpanzees were inoculated by the intranasal and intratracheal routes with the indicated amount of virus in a 1-ml inoculum per site.

<sup>c</sup> Nasopharyngeal swab samples were collected daily for 10 days, and tracheal lavage samples were collected on days 2, 5, 6, 8, and 12. ND, not determined.

<sup>d</sup> Mean peak titers (log<sub>10</sub> PFU/ml) were calculated by using the peak virus titer achieved in each animal.

<sup>e</sup> Data from the study of Whitehead et al. (29).

<sup>f</sup> Historic control animals from the study of Crowe et al. (10).
out mutations to the list, namely, the deletion of NS1 and the silencing of the M2-2 open reading frame.

Among the mutant viruses shown in Table 1, the order of increasing attenuation in seronegative juvenile chimpanzees was rA2ΔSH < rA2ΔNS<2 < rA2cp248/404 < rA2ΔNS<1 < rA2ΔM2-2. All viruses provided similar, high levels of protection against challenge with wild-type RSV (Table 2). Thus, rA2ΔNS<1 and rA2ΔM2-2 each have the desired property of being slightly more attenuated than rA2cp248/404, the recombinant version of cpts248/404, which was slightly too reactogenic in RSV-naïve 1- to 2-month-old infants, as mentioned above (33). The finding that rA2ΔM2-2 is slightly more attenuated than rA2ΔNS<1 increases the chances that one of these viruses will have an optimal level of attenuation. The seronegative juvenile chimpanzee is somewhat less permissive to RSV than the human RSV-experienced adult (17). Thus, a live-attenuated vaccine virus might have prolonged replication in immunocompromised individuals. Thus, it would be desirable to engineer a recombinant vaccine virus to contain attenuating mutations that cannot revert.

Although the major target for an RSV vaccine is the 1- to 2-month-old infant, a second target is the elderly. The cpts248/404 virus candidate, which was insufficiently attenuated in the RSV-naïve infant, was found to be overattenuated in the RSV-experienced adult (17). Thus, a live-attenuated vaccine virus for RSV-naïve infants will need to be more attenuated than one for use in adults. Since the rA2ΔNS<1 and rA2ΔM2-2 viruses are similar to cpts248/404 in their levels of replication, they likely will be too attenuated to be useful as an adult vaccine. However, each virus is appropriate for further evaluation as a pediatric RSV vaccine, either as currently constructed or with the inclusion of a single or a combination of additional attenuating mutations. It should be noted that if either candidate vaccine proves satisfactory, a partner subgroup B candidate can be rapidly generated by replacing the F and G glycoproteins (31).

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References


18. Grosfeld, H., M. G. Hill, and P. L. Collins. 1995. RNA replication by respiratory syncytial virus (RSV) is directed by the N, P, and L proteins; transcription also occurs under these conditions but requires RSV superinfection for efficient synthesis of full-length mRNA. J. Virol. 69:5677–5686.


