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Recombinant Respiratory Syncytial Virus Bearing a Deletion of either the NS2 or SH Gene Is Attenuated in Chimpanzees

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The NS2 and SH genes of respiratory syncytial virus (RSV) have been separately deleted from a recombinant wild-type RSV strain, A2 (M. N. Teng and P. L. Collins, J. Virol. 73:466–473, 1998; A. Bukreyev et al., J. Virol. 71:8973–8982, 1997; and this study). The resulting viruses, designated rA2ΔNS2 and rA2ΔSH, were administered to chimpanzees to evaluate their levels of attenuation and immunogenicity. Recombinant virus rA2ΔNS2 replicated to moderate levels in the upper respiratory tract, was highly attenuated in the lower respiratory tract, and induced significant resistance to challenge with wild-type RSV. The replication of rA2ΔSH virus was only moderately reduced in the lower, but not the upper, respiratory tract. However, chimpanzees infected with either virus developed significantly less rhinorrhea than those infected with wild-type RSV. These findings demonstrate that a recombinant RSV mutant lacking either the NS2 or SH gene is attenuated and indicate that these deletions may be useful as attenuating mutations in new, live recombinant RSV vaccine candidates for both pediatric and elderly populations. The ΔSH mutation was incorporated into a recombinant form of the *cpts*248/404 vaccine candidate, was evaluated for safety in seronegative chimpanzees, and can now be evaluated as a vaccine for humans.

Respiratory syncytial virus (RSV) remains the leading cause of serious viral bronchiolitis and pneumonia in infants and young children throughout the world and accounts for approximately 90,000 hospitalizations in the United States each year (6, 16, 22). RSV infection is also an important cause of severe respiratory illness in elderly (11–13, 24) and immunocompromised (17, 19, 31) populations. While the significance of RSV as a respiratory pathogen has made development of an effective RSV vaccine a public health priority, such a vaccine does not exist. Toward this goal, our laboratory has sought to develop a live, attenuated RSV vaccine since a live virus vaccine, which mimics natural infection, should induce a balanced cellular and humoral immune response without potentiating disease upon subsequent infection with wild-type (*wt*) virus (6, 25).

A number of live attenuated RSV vaccine candidates have been evaluated in animals and humans, and the most promising subgroup A vaccine candidates, *cpts*248/404 and its recombinant counterpart rA2cp248/404, have been shown to possess both temperature-sensitive (*ts*) and non-*ts* mutations which contribute to the attenuation of the virus (8, 14, 33, 34). Our approach toward vaccine development is based on the observation that a combination of *ts* and non-*ts* attenuating mutations in a single virus yields effective vaccine candidates with increased phenotypic stability, as has been seen for influenza virus (26, 27, 30), parainfluenza virus (18), and poliovirus (2, 28, 32). By evaluating several *ts* virus lineages derived from cold-passaged (*cp*) RSV (15), we have identified a sufficient collection of *ts* attenuating mutations (10, 14, 20, 21, 33) and have recently focused our efforts on the identification of stable, non-*ts* attenuating mutations suitable for inclusion in candidate vaccine strains (3, 34).

As the prototype of the pneumovirus genus of the family *Paramyxoviridae*, RSV is an enveloped, negative-sense, single-stranded RNA virus with a genome that is 15,222 nucleotides in length and that encodes 10 subgenomic mRNAs (6). Transcription of viral genes is directed by short, conserved gene start and gene end (GE) *cis*-acting signals that flank each gene and are separated by intergenic regions of various nucleotide lengths. These mRNAs are translated into 11 known proteins: four nucleocapsid proteins, namely, nucleocapsid N protein, phosphoprotein P, large polymerase subunit L, and transcription elongation factor M2-1; three transmembrane envelope glycoproteins, namely, fusion F protein, attachment G protein, and small hydrophobic SH protein; two nonstructural proteins, NS1 and NS2; the matrix M protein; and the putative negative regulatory factor M2-2. Although each of these proteins has been identified, the specific functions of several proteins, such as NS1, NS2, and SH, have not been determined, although NS1 has been shown to inhibit RNA synthesis (1).

By using the previously described reverse genetics system (5), a recombinant *wt* RSV, designated rA2, was generated. It contains the previously described 4C leader mutation, a set of four intergenic and noncoding region marker mutations, and a set of six L gene translationally silent restriction site markers, as well as two F gene mutations required to bring the coding region of the recombinant *wt* clone into agreement with that of the human embryonic kidney (HEK) cell-passaged *wt* RSV (5, 34). Mutant viruses in which either the NS2 or the SH gene (and its respective transcription signal) was completely deleted from rA2 have been successfully engineered, thus demonstrating the dispensable nature of either gene for replication in tissue culture. For deletion of the NS2 gene (ΔNS2), nucleotides 577 to 1098 were removed, which segment joins the authentic NS1 GE sequence to the complete NS2-N intergenic

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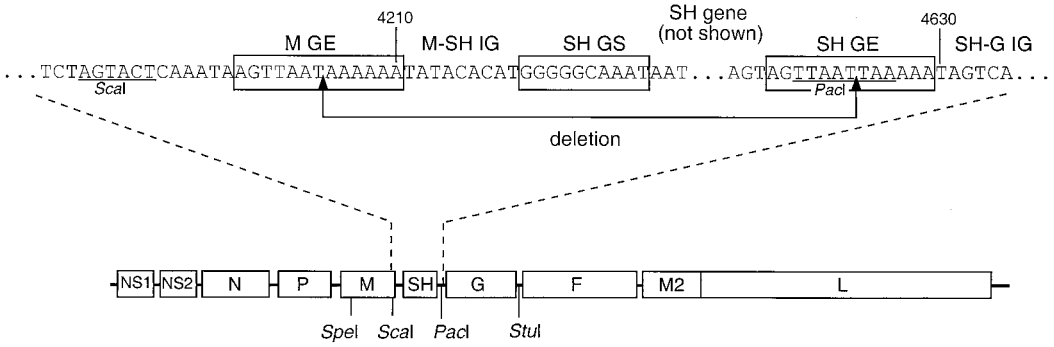

FIG. 1. Deletion of the SH gene to generate rA2ΔSH. The map of the negative-sense *wt* RSV genome is shown (not to scale), as is the sequence illustrating the deletion, which involved nucleotides 4205 to 4623 (arrows). To construct rA2ΔSH, plasmid D50, containing the left-hand end of the genome from the 3′ leader region to the end of the M2 gene, was digested with *Sca*I and *Pac*I and the resulting fragment was replaced with a short double-stranded DNA constructed by hybridizing the two synthetic oligonucleotides ACTCAAATAAGTTAAT and TAACTTATTTGAGT. In the final construction, the deletion extended from the middle of the M GE signal to the middle of the SH GE signal. In the ΔSH genome, compared to its *wt* recombinant parent, the M GE signal sustained a single nucleotide change (AGTTAATAAAAAA to AGTTAATTAAAAA, with the change underlined) to become identical to that of the *wt* SH GE signal. The modified D50 plasmid was then used to assemble a complete antigenome by ligation with a cDNA containing the L gene and trailer region as described previously (5, 33, 34). IG, intergenic region; GS, gene start signal; GE, gene end signal.

region (29). For deletion of the SH gene (ΔSH), the *Sca*I-*Pac*I restriction fragment containing the SH gene was deleted and replaced with a short synthetic DNA, resulting in the removal of nucleotides 4205 to 4623 (Fig. 1). This process introduced a single nucleotide change in the M GE signal, making it identical to that of the *wt* SH GE signals. It should be noted that the ΔSH virus described here (Fig. 1) is different from the one designated D46/6368, which was described previously (3) and which lacks the six silent L gene restriction sites and the two F gene mutations noted above. In chimpanzees, neither the L gene restriction sites nor the F gene mutations affected the replication of recombinant RSV (34). Also, D46/6368 contains some incidental, heterologous sequence and an unusually long intergenic region at the deletion point. Like their *wt* recombinant parent, the rA2ΔSH and rA2ΔNS2 viruses contain only authentic transcription signals and intergenic regions. Also, they have the same genetic background as previously described biologically derived and recombinant *cpts* vaccine candidates and thus can be compared directly with those vaccine candidates.

The NS2 and SH deletion viruses have been characterized with regard to their growth phenotypes in cell culture. On HEp-2 cell monolayers, D46/6368 grew slightly better than *wt* RSV (up to 12.6-fold higher levels) and consistently produced plaques that were 70% larger (reference 3 and data not shown), while the rA2ΔNS2 virus grew more slowly than *wt* RSV and produced pinpoint plaques at a titer 5- to 50-fold lower than that of *wt* RSV (29). When inoculated intranasally into mice, the D46/6368 virus resembled *wt* RSV in its level of replication in the lungs, whereas it replicated to a level 10-fold lower in the upper respiratory tract (3). Also in mice, D46/6368 and *wt* RSV were similar with respect to immunogenicity and efficacy in inducing resistance to RSV challenge (3). In addition, the SH gene was also deleted from the D53cp248/404 cDNA, which encodes a recombinant version of the *cpts*248/404 vaccine candidate. This deletion was made by replacing the *Spe*I-*Stu*I cassette of D53cp248/404, which contains the SH gene, with the same cassette from the ΔSH cDNA (Fig. 1). The resulting virus, rA2cp248/404ΔSH, maintained the same level of temperature sensitivity as rA2cp248/404 and acquired a larger plaque size at the permissive temperature (data not shown). If the ΔSH and ΔNS2 mutant viruses exhibit an attenuation (*att*) phenotype in chimpanzees, these mutants would be of special interest for vaccine development, since it is expected that a high level of genetic stability in vitro and in vivo would be afforded by complete deletion of a viral gene. Satisfactorily attenuated and immunogenic mutants of this type might be especially safe for use in immunocompromised subjects.

The replication of the rA2ΔNS2 and rA2ΔSH deletion mutants was evaluated in a separate study with young RSV-seronegative chimpanzees according to established procedures (9), and their levels of replication and reactogenicity were compared with those of rA2 and rA2cp248/404. These findings were also compared to results from previous studies with *wt* RSV, which was shown to produce acute respiratory tract disease in humans and chimpanzees (7, 9, 23). Chimpanzees are the only known nonhuman host in which RSV replication and virulence approach that observed in seronegative humans. These features are indispensable for evaluation of a live attenuated RSV prior to administration to humans, particularly with mutants containing novel gene deletions. Because of the very limited number of RSV-seronegative chimpanzees available for study, the sizes of the experimental groups were small. Animals were inoculated simultaneously by the intranasal and intratracheal routes. Upper respiratory tract (nasopharyngeal swab) and lower respiratory tract (tracheal lavage) samples were collected over a period of 10 days, and the chimpanzees were monitored daily for symptoms of rhinorrhea. To compare the levels of virus replication in the animal groups, we determined the mean peak titers of infectious virus in both the upper and lower respiratory tracts. We considered differences in mean peak titers greater than 10-fold to be significant, which was confirmed statistically by the use of Duncan's Multiple Range test. Rhinorrhea scores (see Table 1, footnote *d*, for a definition) were also compared, and we considered scores greater than 1.0 to be significant based on extensive prior experience with this experimental model of RSV upper respiratory tract disease. The two *wt* viruses, RSV A2 and rA2, were comparable in their levels of virus replication and in the extents of rhinorrhea that they caused in chimpanzees (Table 1), despite the 21 nucleotide differences between the two viruses, which represent the nucleotide changes engineered into rA2 (5, 34). Since rA2 replicates and induces rhinorrhea in a manner similar to that of its biologically derived counterpart, it is an appropriate virus to compare with the rA2ΔSH and

TABLE 1. Deletion of either the NS2 or the SH gene attenuates RSV for chimpanzees

Virus used to infect chimpanzees[a]	No. of animals	Dose[b] (per site, $\log_{10}$ PFU)	Mean peak virus titer ($\log_{10}$ PFU/ml) (Duncan grouping)[c] in:		Rhinorrhea score[d] (range, 0–4)		Mean titer of neutralizing antibody in serum (reciprocal $\log_2$)[e] on day:	
			Nasopharyngeal swab specimen	Tracheal lavage fluid	Peak	Mean	0	28
wt RSV A2	2[f]	4.0	5.0 ± 0.35 (A)	5.5 ± 0.40 (A)	3.0	1.4	<3.3	11.2
rA2	2	4.0	4.9 ± 0.15 (A)	5.4 ± 0.05 (A)	2.5	1.3	<3.3	10.5
rA2ΔSH	3	4.0	4.6 ± 0.10 (A, B)	3.8 ± 0.31 (B)	1.0	0.5	<3.3	10.2
rA2ΔNS2	4	4.0	3.8 ± 0.41 (B)	1.4 ± 0.29 (C)	1.0	0.4	3.4	10.6
rA2cp248/404	4	5.0	2.5 ± 0.25 (C)	1.4 ± 0.37 (C)	0.8	0.1	3.4	10.6
rA2cp248/404ΔSH	4	5.0	2.1 ± 0.13 (C)	1.5 ± 0.40 (C)	0.8	0.2	3.4	10.3

[a] All recombinant viruses (r) contain the sites and HEK mutations previously described (34).
[b] Chimpanzees were inoculated by the intranasal and intratracheal routes with the indicated amounts of virus in a 1-ml inoculum per site. Nasopharyngeal swab samples were collected daily for 10 days, and tracheal lavage samples were collected on days 2, 5, 6, 8, and 10.
[c] Mean virus titers were assigned to statistically similar groups by Duncan's Multiple Range test ($\alpha = 0.05$). Therefore, means in each column with different letters are significantly different.
[d] The amount of rhinorrhea was estimated daily and assigned a score (0 to 4) that indicated extent and severity. Scores indicate severe (score, 4), moderate (score, 3), mild (score, 2), a trace amount of (score, 1), or no (score, 0) rhinorrhea. Mean rhinorrhea scores are the sums of scores during the 8 days of peak virus shedding divided by 8.
[e] Serum RSV-neutralizing antibody titers were determined by a complement-enhanced 60% plaque reduction assay with *wt* RSV A2 and HEp-2 cell monolayer cultures incubated at 37°C (4). RSV-seronegative chimpanzee serum used as a negative control had a neutralizing antibody titer of <3.3 $\log_2$ reciprocal. Adult human serum used as a positive control had a neutralizing antibody titer of 11.4 $\log_2$ reciprocal.
[f] Historic control animals from the study of Crowe et al. (9).

rA2ΔNS2 mutants, which are isogenic with it except for deletion of an RSV gene.

Mutant virus rA2ΔNS2 was 12.5-fold reduced in replication in the upper respiratory tract compared to rA2 (Table 1), whereas rA2ΔSH replicated to a level comparable to that of rA2. However, in the lower respiratory tract, rA2ΔNS2 and rA2ΔSH showed 10,000-fold and 40-fold reductions in replication, respectively, compared to the level of replication of rA2. In comparison with chimpanzees receiving either *wt* RSV A2 or rA2, chimpanzees infected with rA2ΔNS2 or rA2ΔSH exhibited less rhinorrhea. These findings demonstrate that deletion of either the NS2 or the SH gene leads to attenuation of both replication and disease symptoms in chimpanzees, with deletion of the NS2 gene having a greater effect.

Immunization with either rA2ΔNS2 or rA2ΔSH induced a high level of neutralizing antibodies that was comparable to that induced by infection with either of the *wt* control viruses (Table 1). Immunization with rA2ΔNS2, as with *cpts*248/404, induced resistance to replication of *wt* RSV in both the upper and lower respiratory tracts (Table 2). Taken together, these results indicated that immunization with rA2ΔNS2 was nearly as effective as immunization with mutant *cpts*248/404, even though *cpts*248/404 was administered at a fivefold higher dose. Because the ΔSH mutant D46/6368 was shown previously to induce complete protection in mice against *wt* RSV challenge (3), this analysis was not repeated with chimpanzees.

Vaccine candidate *cpts*248/404 caused a significant level of nasal congestion in a majority of vaccinees in the RSV vaccine target population of one-month-old infants, which suggests that it is incompletely attenuated for this age group (35). Therefore, more attenuated derivatives of *cpts*248/404 will be needed for use in very young infants. Since neither rA2ΔNS2 nor rA2ΔSH is as attenuated in chimpanzees as the incompletely attenuated rA2cp248/404 vaccine candidate, the ΔNS2 and ΔSH mutants are of interest for use in conjunction with the mutations present in rA2cp248/404. We have initiated studies designed to further attenuate *cpts*248/404 by the deletion of the SH gene, the less attenuating of the two deletion mutations, from rA2cp248/404. This mutation was selected for study first since the clinical evaluation of *cpts*248/404 in young infants

TABLE 2. Recombinant virus rA2ΔNS2 is highly protective against challenge with *wt* RSV A2 in the upper and lower respiratory tracts of chimpanzees[a]

Immunizing virus	Inoculum dose ($\log_{10}$ PFU)	No. of chimpanzees	Mean virus titer following RSV challenge ($\log_{10}$ PFU/ml) in:						Peak rhinorrhea score[b]
			Nasopharyngeal swab specimen on day:			Tracheal lavage fluid on day:			
			3	5	7	3	5	7	
rA2ΔNS2	4.0	4	1.3	1.4	1.1	0.9	2.2	1.0	1.0
*cpts*248/404	4.7	2[c]	2.3	1.4	0.8	<0.7	<0.7	<0.7	1.0
None		2[c]	4.0	5.0	3.7	1.7	4.6	4.2	3.0

[a] Chimpanzees were inoculated by the intranasal and intratracheal routes with the indicated amounts of virus in a 1-ml dose per site. After 56 days, chimpanzees were administered 4.0 $\log_{10}$ PFU of *wt* RSV A2 by the intranasal and intratracheal routes. Nasopharyngeal swab and tracheal lavage samples were then collected after 3, 5, and 7 days.
[b] The amounts of rhinorrhea were estimated daily and assigned a score (0 to 4) that indicated extent and severity. See Table 1, footnote *d*, for an explanation of the scores.
[c] Historic control animals from the study of Crowe et al. (9).

indicated that only a slight increase in attenuation would be necessary to produce a satisfactory candidate vaccine and because the SH deletion was associated with a significant reduction in rhinorrhea (Table 1). The SH gene was removed from rA2cp248/404, and the resulting virus, rA2cp248/404ΔSH, was administered to chimpanzees (Table 1). Since *cpts*248/404 is highly attenuated in chimpanzees at a dose of 4.0 $\log_{10}$ PFU/ml (8), its recombinant derivatives rA2cp248/404 and rA2cp248/404ΔSH were administered at an elevated dose of 5.0 $\log_{10}$ PFU/ml in an attempt to augment their levels of replication to levels that would permit the detection of a difference in growth capacity. As shown in Table 1, a significant difference in levels of replication of the two viruses in the upper and lower respiratory tracts was not observed and a difference in the levels of rhinorrhea or in the neutralizing antibody responses in sera was not observed. In rA2cp248/404ΔSH, the attenuation conferred by deletion of the SH gene may be masked by the *cpts*248/404 *att* mutations. An increase in attenuation of rA2cp248/404ΔSH may be apparent only in humans, where *cpts*248/404 replicates to a 100-fold higher titer than in chimpanzees (35), and such studies have been initiated. Since the attenuating mutations identified in *cpts*248/404 are point mutations, it is hoped that the overall stability of the attenuation phenotype of rA2cp248/404 will be increased by the complete deletion of the SH gene.

Deletion of NS2 is the single most attenuating non-*ts* mutation we have studied to date, significantly surpassing the attenuation conferred by the *cp* and ΔSH mutations in both the upper and the lower respiratory tract (34). Mutant virus rA2ΔNS2 replicates slightly better than rA2cp248/404 in the upper respiratory tracts of chimpanzees, and this property suggests a possible role for it as a vaccine candidate for the elderly. To be effective, a live attenuated vaccine for this group would need to be capable of replicating and immunizing in the presence of RSV antibodies induced by prior infections and yet be sufficiently attenuated in the lower respiratory tract so as to preclude any serious reactogenicity. Because the rA2ΔNS2 virus was highly attenuated in the lower respiratory tracts of chimpanzees yet remained relatively infectious for the upper respiratory tracts, this virus may have potential use as a vaccine for a seropositive population. In addition, the feasibility of removing the NS2 gene from rA2cp248/404 and other attenuated recombinant viruses for use in the pediatric population is also under investigation in our laboratory.

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