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Recombinant Respiratory Syncytial Virus (RSV) Bearing a Set of Mutations from Cold-Passaged RSV Is Attenuated in Chimpanzees

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A set of five missense mutations previously identified by nucleotide sequence analysis of subgroup A cold-passaged (*cp*) respiratory syncytial virus (RSV) has been introduced into a recombinant wild-type strain of RSV. This recombinant virus, designated rA2cp, appears to replicate less efficiently in the upper and lower respiratory tracts of seronegative chimpanzees than either biologically derived or recombinant wild-type RSV. Infection with rA2cp also resulted in significantly less rhinorrhea and cough than infection with wild-type RSV. These findings confirm the role of the *cp* mutations in attenuation of RSV and identify their usefulness for inclusion in future live attenuated recombinant RSV vaccine candidates.

Infection with respiratory syncytial virus (RSV) remains the most common cause of serious viral bronchiolitis and pneumonia in infants and children worldwide. The age distribution of serious RSV-associated illness is unusual among the respiratory tract pathogens in that disease occurs most commonly during the first several months of life, despite the presence of maternally transmitted serum neutralizing antibodies (5). Respiratory tract disease occurs less frequently and is generally less severe during reinfection with RSV than following first infection (5). Furthermore, the initial infection with RSV confers a significant degree of protection, and serum and local antibodies have been found to be mediators of immunity to RSV (5). The primary goal of immunization with an RSV vaccine is to prevent the severe lower respiratory tract disease associated with first and second infections with RSV. Live attenuated RSV vaccines represent promising vaccine candidates for this purpose since (i) they can efficiently immunize in the presence of passively acquired RSV antibodies which are present in the target population, the very young infant (9); (ii) they induce both serum immunoglobulin G and local immunoglobulin A antibodies; and (iii) they do not cause immune response-mediated disease enhancement like that seen following immunization with formalin-inactivated RSV (3).

Since early work indicated that attenuated mutants of poliovirus and measles virus could be selected by growth at low temperature (20, 21), a cold-passaged RSV (*cp*RSV) subgroup A candidate was produced from the A2 strain of RSV by 52 passages in bovine embryonic kidney tissue culture at progressively lower temperatures, the final and lowest temperature being 26°C (12). *cp*RSV was shown to be completely attenuated in seropositive adults and children but still caused some moderate respiratory tract disease in RSV-seronegative infants (12, 16, 18). These studies with humans indicated that the biologically derived *cp*RSV vaccine candidate possessed an attenuation phenotype, and this was subsequently confirmed in seronegative chimpanzees (8). *cp*RSV lacks an in vitro marker of attenuation since the virus is neither significantly cold adapted nor temperature sensitive (*ts*) in tissue culture (6, 8, 12) and therefore exhibits the host range phenotype; i.e., its replication is permissive in tissue culture but restricted in humans and chimpanzees. The chimpanzee is the only experimental animal that can be used to assess this attenuation phenotype.

To better understand the genetic basis for the attenuation of *cp*RSV, the sequence of the entire genome was determined and compared to that of wild-type (wt) RSV strain A2 (6). At the time this sequencing project was initiated, the exact parent of *cp*RSV, which had been passaged five times in human embryonic kidney (HEK) tissue culture, no longer existed, but a derivative which had been further passaged two times in HEK tissue culture (HEK-7) was available. Sequence analysis of *cp*RSV and RSV HEK-7 showed that *cp*RSV had accumulated five nucleotide substitutions, one in the N gene, two in the F gene, and two in the L gene, resulting in five predicted amino acid substitutions (6, 10) (Table 1). The recent development of a system for recovery of infectious virus from cDNA clones of RSV permits us to identify the genetic basis of attenuation of RSV vaccine candidates, as well as to develop new vaccine candidates (4). Therefore, the goal of the present study was to determine if the set of five *cp* mutations, which have been identified by sequence analysis, indeed confers the attenuation phenotype. This is of particular importance because *cp*RSV was the parent for a series of further-attenuated RSV strains (described below), and thus the *cp* mutation set is a common feature of current RSV vaccine candidates.

The five *cp* mutations were introduced together into a modified cDNA representing the RSV subgroup A genome by using our previously described methods (15). The antigenome cDNA that was the starting material for these experiments was cDNA D46, from which RSV was first recovered in 1995 (4). cDNA D46 differed in two ways from the natural A2 isolate from which the cDNA was prepared. First, four restriction enzyme cleavage sites (marker mutations) (Table 1) were created by a single nucleotide insertion in the NS2-N intergenic region (IGR) and five nucleotide substitutions, two in the N-gene noncoding region, two in the G-F IGR, and one in the F-M2 IGR (4). Second, a G-to-C (negative-sense) substitution at nucleotide 4 in the leader region (the 4C mutation)

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TABLE 1. Mutations introduced into full-length cDNA clones used to create recombinant RSV

Mutation	Gene	Nucleotide position[a]	Restriction marker (cleavage site position)	Sequence changes[b]	Amino acid position	Amino acid change
4C	Leader			A2 wt: ACG**C**GAAAAAATGCGTAC D46: ACG**G**GAAAAAATGCGTAC		Noncoding
Marker	NS2-N IGR		*Afl*II (1099)	A2 wt: AATTTAAAA.TTAAGGAG D46: AATTTAAAA**C**TTAAGGAG		Noncoding
	N NCR		*Nco*I (1139)	A2 wt: CAAATACAA**AG** ATG GCT D46: CAAATACAA**CC** ATG GCT		Noncoding
	G-F IGR		*Stu*I (5613)	A2 wt: ACAAAA**A**GCC**A**TGACCAA D46: ACAAAA**G**GCC**T**TGACCAA		Noncoding
	F-M2 IGR		*Sph*I (7563)	A2 wt: CACAATTG**A**ATGCCAGAT D46: CACAATTG**C**ATGCCAGAT		Noncoding
Site	L		*Bsu*36I (9399)	A2 wt: TTA GGC **T**TA AG**A** TGC rA2sites: TTA GGC **C**TA AG**G** TGC		Silent
	L		*Sna*BI (11848)	A2 wt: GAA CCT AC**A** TAT CCT rA2sites: GAA CCT AC**G** TAT CCT		Silent
	L		*Pme*I (13342)	A2 wt: AAA CGT **CTT** AA**T** GTA rA2sites: AAA CGT **TTA** AA**C** GTA		Silent
	L		*Rsr*II (14083)	A2 wt: TTG CG**T** AC**A** GTA GTG rA2sites: TTG CG**G** AC**C** GTA GTG		Silent
	L		*Bst*EII (14318)	A2 wt: TTG TC**T** GTA AC**A** GTC rA2sites: TTG TC**G** GTA AC**C** GTC		Silent
	L		*Sna*BI (14477)	A2 wt: AAA ACT TA**T** GTA TGC rA2sites: AAA ACT TA**C** GTA TGC		Silent
HEK	F	5857	*Ssp*I (5850)	D46: AGT AAT AT**C** AAG *Å**A**A HEK-7: AGT AAT AT**C** AAG **G**AA rA2cp: AGT AAT AT**T** AAG **G**AA	66	Lys→Glu
	F	5963	*Sca*I (5958)	D46: CAA AG**C** AC**A** C*Å**A** GCA HEK-7: CAA AG**C** AC**A** C**C**A GCA rA2cp: CAA AG**T** AC**T** C**C**A GCA	101	Gln→Pro
cp	N	1939	*Cla*I (1936)	A2 wt: GCA AAA TC**A** *G**T**T AAA rA2cp: GCA AAA TC**G** A**T**T AAA	267	Val→Ile
	F	6314	*Nru*I (6313)	A2 wt: TCA AAT AT**A** G*A**A** ACT rA2cp: TCA AAT A**TC** G**CG** ACT	218	Glu→Ala
	F	7229	*Ase*I (7229)	A2 wt: TCC ACC A*C**A** AAT ATC rA2cp: TCC ACC A**TT** AAT ATC	523	Thr→Ile
	L	9454	Lose *Acc*I (9455)	A2 wt: GGA GAT T*G**T** ATA CTA rA2cp: GGA GAT T**AC** ATA CTA	319	Cys→Tyr
	L	13566	*Hpa*I (13558)	A2 wt: CT**A** TTA ACT AAA *C**A**T rA2cp: CT**G** TTA ACT AAA T**AC**	1690	His→Tyr

[a] The nucleotide position shown is for nucleotide changes (indicated by an asterisk) leading to an amino acid substitution in the biologically derived wt RSV (HEK-7) and *cp*RSV. Numbering reflects the one nucleotide insertion in the NS2-N IGR of recombinant virus.

[b] Nucleotide changes (positive sense) are shown in boldface type. Restriction enzyme recognition sites are underlined.

(Table 1) was introduced since C at this position was previously found to be an up-regulator of RNA synthesis in an RSV minigenome system (13, 19a). This G-to-C substitution does not have an effect on the level of attenuation specified by a candidate RSV vaccine (11), an observation which is also confirmed here. Infectious virus recovered from this cDNA is designated rD46. cDNA D46 was then modified by the insertion of a set of six translationally silent restriction enzyme

TABLE 2. The set of five *cp* mutations attenuates RSV for the upper and lower respiratory tracts of chimpanzees

Virus used to infect animal[a]	Marker				Chimpanzee no.[b]	Mean virus titer (log_{10} PFU/ml)				Rhinorrhea score[d]		Days with cough
						Nasal wash sample		Tracheal lavage sample				
	4C	Site	HEK	*cp*		Peak titer	Daily titer[c]	Peak titer	Daily titer[c]	Peak	Mean	
rD46	X				1	4.9	3.7	3.4	3.2	3	1.1	4
	X				2	5.1	3.1	4.6	4.5	4	2.0	4
	X				3	4.4	3.9	4.0	2.5	3	1.4	5
	X				4	4.6	3.5	4.7	3.0	4	2.6	5
Mean ± SE						4.8 ± 0.16	3.6 ± 0.15	4.2 ± 0.30	3.1 ± 0.33	3.5	1.8	4.0
A2 wt[e]			X		5*	4.6	3.6	5.1	3.7	3	1.0	2
			X		6*	5.3	3.6	5.9	4.0	3	2.1	0
Mean ± SE						5.0 ± 0.35	3.6 ± 0.30	5.5 ± 0.40	3.8 ± 0.46	3.0	1.6	1.0
rA2sites	X	X			7	4.8	4.2	4.1	3.7	2	0.9	1
	X	X			8	5.4	3.8	5.5	4.1	3	1.4	3
	X	X			9	4.8	4.4	5.3	4.2	2	0.9	3
	X	X			10	4.9	3.7	3.8	2.9	3	2.0	3
Mean ± SE						5.0 ± 0.14	4.0 ± 0.18	4.7 ± 0.43	3.7 ± 0.33	2.5	1.3	2.5
rA2cp	X	X	X	X	11	4.3	2.1	4.5	3.3	2	1.1	0
	X	X	X	X	12	3.8	2.7	1.0	1.0	2	0.9	0
	X	X	X	X	13	4.6	3.2	3.7	3.2	0	0	0
	X	X	X	X	14	4.8	3.5	3.8	2.4	0	0	0
Mean ± SE						4.4 ± 0.22	2.9 ± 0.23	3.3 ± 0.77	2.7 ± 0.40	1.0	0.5	0.0
*cp*RSV[f]			X	X	15*	5.0	3.6	2.8	2.8	1	0.5	0
			X	X	16*	4.3	3.4	3.0	3.0	1	0.6	0
Mean ± SE						4.7 ± 0.35	3.5 ± 0.22	2.9 ± 0.10	2.9 ± 0.10	1.0	0.6	0.0

[a] Chimpanzees were inoculated by the intranasal and intratracheal routes with 10^4 PFU of the indicated virus in a 1-ml dose per site. Nasal wash samples were collected daily for 10 days, and tracheal lavage samples were collected on days 2, 5, 6, 8, and 10.

[b] *, historic control animal from the work of Crowe et al. (7).

[c] Mean daily titers were computed for each chimpanzee by adding together the virus titers from days with detectable levels of virus (≥0.7 PFU/ml) and dividing this sum by the total number of days with detectable virus titer.

[d] 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. Mean rhinorrhea scores represent the sum of scores during the 8 days of peak virus shedding divided by 8.

[e] Biologically derived control virus (lot F-059) for which the presence of the HEK F-gene mutations was confirmed.

[f] Biologically derived *cp* virus (lot 3131).

cleavage sites (site mutations) (Table 1) which were created in the L gene to act as genetic markers for identification of recombinant virus and to aid in future construction of L-gene mutants. This cDNA, designated D53sites, was used to generate the recombinant RSV rA2sites (15). Finally, the set of five *cp* mutations was introduced together with two additional mutations (HEK mutations) (Table 1) required to bring the F-gene coding region of the recombinant virus into agreement with that of RSV HEK-7. These two changes in F were made because alignment of the sequences of cDNA D46 and the HEK-passaged strain A2 RSV revealed two predicted amino acid substitutions in the F protein (6). These two F-gene changes are the only significant differences between the two passage levels of the RSV A2 isolates: the RSV A2 derivative, HEK-7, which has remained in a freezer for most of the last three decades, and the further-passaged RSV A2 that is in use in our laboratory, which is related to HEK-7 but which has undergone numerous rounds of plaque purification and propagation predominantly in HEp-2 cells. It is thus remarkable that the two viruses have so few differences. With the introduction of the two coding changes in F, the cDNA-encoded virus is identical at the amino acid level to HEK-7. Each of the *cp* and the two HEK mutations introduced into cDNA were genetically marked by an accompanying translationally silent restriction enzyme cleavage site addition or deletion. Virus recovered from this cDNA was designated rA2cp and contained the original 4C and marker mutations found in rD46, the six L-gene restriction site markers, the two HEK F-gene mutations, and the set of five *cp* mutations. The rD46, rA2sites, and rA2cp mutants, like their biologically derived counterparts, produced plaques efficiently on monolayers of tissue culture cells at 40°C and therefore are non-*ts* viruses.

The attenuation phenotype of rA2cp was evaluated in RSV-seronegative chimpanzees as previously described (8) by comparing its levels of replication and pathogenicity with those of the rD46 and rA2sites wt recombinant viruses (Table 2). These findings, in turn, were compared to results from previous control studies with biologically derived *cp*RSV and wt RSV A2. Because of the severely limited number of available RSV-seronegative chimpanzees, test groups in this study were rela-

tively small and limited to four animals. Upper respiratory tract (nasal wash) 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 and cough. To compare the levels of virus replication of wt and attenuated viruses, we determined mean peak titers and mean daily titers. Whereas the peak titers compare the maximum levels of virus replication achieved in each animal, the mean daily titers (see Table 2, footnote *c*, for definition) estimate the total extent of replication. We generally consider differences in mean titer greater than 10-fold as significant. Rhinorrhea scores (see Table 2, footnote *d*, for definition) and cough symptoms were also compared. We have defined mean rhinorrhea scores greater that 1.0 as significant and consider any day with coughing as significant. The three wt viruses, rD46, rA2sites, and biologically derived A2 wt, were comparable in their levels of virus replication and in the extent of illness they caused in chimpanzees (Table 2). Likewise, the levels of virus replication and illness in chimpanzees infected with rA2cp and the biologically derived *cp*RSV were similar. This is so despite the 28 nucleotide differences between the two viruses, which represent the silent changes purposefully introduced into rA2cp. Specifically, the mean peak or daily virus titers in either the upper or lower respiratory tract did not appear to be significantly different between rA2cp and biologically derived *cp*RSV. Importantly, each of the two *cp* viruses replicated less well and induced fewer symptoms than the wt viruses.

The rA2cp virus was directly compared with its most closely related recombinant wt virus, rA2sites. In the lower respiratory tract, the rA2cp mutant virus exhibited a 25-fold decrease in peak virus titer as well as a 10-fold decrease in mean daily virus titer compared to the titers for the rA2sites virus. In the upper respiratory tract, there were also modest 4- and 10-fold decreases in the peak virus titer and mean daily titer, respectively. In comparison with chimpanzees receiving wt rA2sites, those inoculated with rA2cp showed a marked decrease in rhinorrhea and cough. These findings with rA2cp demonstrate that the set of five *cp* mutations indeed specifies the host range, attenuation phenotype. Unfortunately, due to the limited availability of RSV-seronegative chimpanzees, it is not feasible at this time to examine the contribution of individual *cp* mutations to overall virus attenuation and prevention of illness associated with infection.

The design of these studies also allows for an evaluation of the effects of the other sets of introduced mutations on virus replication or illness. The original recombinant virus, rD46, was comparable in replication and virulence to the biologically derived A2 wt virus (Table 2, compare results for chimpanzees 1 to 4 with chimpanzees 5 and 6). This shows that this recombinant virus, on which all subsequent engineered viruses will be based, indeed is wt with respect to replication and virulence in a fully permissive experimental animal. Thus, it does not contain any incidental deleterious changes, and furthermore the four marker mutations and the 4C mutation do not significantly alter its properties or that of its rA2cp derivative. In addition, since the mean virus titers of rA2sites and rD46 are not significantly different, the six translationally silent L-gene site mutations do not appear to affect virus replication in this permissive host (Table 2, compare results for chimpanzees 1 to 4 with chimpanzees 7 to 10). Similarly, the HEK F-gene mutations do not appear to modify virulence (Table 2, compare results for chimpanzees 5 and 6 with chimpanzees 1 to 4 and 7 to 10). However, since the HEK-7 RSV was the genetic background for biologically derived *cp*RSV, it is possible that the *cp* mutations may interact with these HEK F-gene mutations. Since this is not directly tested here, the HEK F-gene mutations will be included with the *cp* mutations in future constructs.

Our approach to the development of a live attenuated vaccine virus is to sequentially introduce both *ts* and non-*ts* attenuating mutations into the genome of wt RSV until a proper balance between attenuation and immunogenicity has been achieved. The rationale for this design is based on the observation that several successful, live attenuated vaccines and vaccine candidates, including those for polioviruses, orthomyxoviruses, and paramyxoviruses, have *ts* mutations accompanied by non-*ts* mutations, both of which contribute to their attenuation (1, 8, 14, 17, 22, 23, 25). Because several live attenuated candidate vaccines that contain only *ts* mutations contributing to their attenuation readily undergo loss of their temperature sensitivity in animals or humans (7, 19, 22, 24), it was considered prudent to stabilize the *ts* and attenuation phenotypes of candidate RSV vaccines by combining both *ts* and non-*ts* attenuating mutations. Since the set of five *cp* mutations indeed specifies the attenuation phenotype, it represents our first non-*ts* attenuating genetic element for RSV. A second non-*ts* attenuating mutation, the deletion of the small hydrophobic (SH) protein of RSV, has also recently been identified (2). We are in the process of identifying the genetic basis of the attenuation and temperature sensitivity of a panel of *ts* viruses, such as *cpts*-248/404, *cpts*-530/1009, and *cpts*-530/1030, which were derived from *cp*RSV and show a range of phenotypes with regard to temperature sensitivity and attenuation (7, 8, 9, 11, 15). The *ts* mutations identified in these studies and the non-*ts* SH deletion mutation are currently being added individually and in combination to rA2cp to assess their contribution to attenuation and to create a new generation of novel live attenuated virus vaccine candidates. In this way, we believe that it will be possible to derive a satisfactorily attenuated and immunogenic RSV vaccine candidate in the near future.

Nucleotide sequence accession number. The nucleotide sequence of rA2cp has been submitted to the GenBank nucleotide sequence database and assigned accession no. AF035006.

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