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Comparative Studies of Wild-Type and Cold-Mutant (Temperature-Sensitive) Influenza Viruses: Nonrandom Reassortment of Genes during Preparation of Live Virus Vaccine Candidates by Recombination at 25° between Recent H3N2 and H1N1 Epidemic Strains and Cold-Adapted A/Ann Arbor/6/60

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Genetic compositions of 35 recombinant cold-adapted influenza A(H3N2 and H1N1) candidate live attenuated vaccine strains have been determined. The viruses, which had been obtained by recombination (reassortment) at 25° between contemporary epidemic wild-type strains and cold-adapted A/Ann Arbor/6/60(H2N2), followed by selection for growth at 25° of virus with wild-type HA and NA, have a highly restricted genetic composition. Eighteen of the thirty-five recombinants had RNAs coding for the three polymerase (P) proteins, NP, M, and NS, from the cold-adapted mutant A/Ann Arbor/6/60 and had only the HA and NA of the wild-type strains. Only 4 out of 64 theoretically possible combinations of genes coding for nonglycoprotein viral products were detected. The restricted genetic composition of cold-adapted recombinants produced at 25° supports the evaluation of this method of preparing live vaccine strains to determine whether recombinants with constant gene composition have predictable levels of attenuation for man.

One method being evaluated for producing live vaccine candidates from new strains is recombination (reassortment) of wild-type (wt) influenza virus isolates with temperature-sensitive (ts) and/or cold-adapted (ca) viruses having desirable levels of immunogenicity and attenuation (*1–3*). We have been analyzing biochemically and biologically a series of recombinants made by using as one parent an A/Ann Arbor/6/60 mutant strain which is believed to have been attenuated during adaptation to replicate at a temperature of 25° (*4*). One objective is to determine the gene composition of recombinants prepared for use in vaccine trials. Such information is required in order to develop an understanding of whether attenuation of the viruses for man is due to the presence in them of specific genes (or clusters of genes) from the cold-adapted parent virus, or is fortuitous, resulting from generation by chance of a gene constellation that reduces virulence of the wt virus, as has been observed in previous studies of human and animal influenza (*5–7*). Initially, we developed methods for identifying the NS, M, NP, HA, and NA genes in A/Ann Arbor/6/60 recombinants (*8, 9*) and observed that ca recombinants from five experiments possessed NP and M genes from the ca parent. Later, we reported electrophoretic methods that also allowed identification of the three large RNAs in five ca recombinants and found that only RNA 2 was ever derived from wt virus (*10*). These findings suggested that the genetic composition of ca recombinants might be restricted so that two large RNAs, as well as the NP and M protein genes were always derived from the ca mutant parent. To investigate this more critically we have prepared and analyzed a larger number of

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independent clones of ca recombinants using as the wt parents contemporary H3N2 and H1N1 strains of influenza A.

Methods used for preparation of ca recombinants were similar to those previously described (*1*, *2*, *8*), involving mixed infection of chick kidney (CK) cells with about 5 PFU/cell of each of two viruses, the A/Ann Arbor/6/60(H2N2) mutant parent (*4*) and a wt H3N2 or H1N1 isolate (Table 1). Cells were incubated at 25° for several days until cytopathic effects were observed and the tissue culture fluid was then used as a source of ca recombinants containing wt H3 or H1 antigen. Selection for such recombinants was accomplished by two sequential passages in CK cells at 25° in the presence of antiserum to the A/Ann Arbor/6/60 virus, followed by plaque purification in CK cells at 25° in the absence of serum. Selection of clones was by two methods. In method 1, virus clones were selected from plaques seen at the last stage of the above-described series of passages which had been initiated with the harvest from a single recombinational mixture. Such viruses might not, therefore, be fully independent clones but reisolations of identical recombinants (*8*). In method 2, the initial recombination was performed multiply in separate tubes of CK cells, and the yield from each tube was then passaged in parallel following the same steps outlined above. After plaque purification a single clone was picked from each of the independent series of parallel recombinants. The method used and parent wt virus for each recombination experiment is described in Table 1. All wt viruses failed to replicate at 25° when passaged in CK cells in parallel to the recombinant mixtures.

All clones recovered from mixed infections were ca and contained the HA of their wt parent, strongly suggesting on the basis of their biological properties that they were recombinants. Occasionally clones were detected that had the neuraminidase of their mutant parent, consistent with the known low neutralizing activity of antineuraminidase antibody. Identification of other genes in the recombinants was done using previously described polyacrylamide gel electrophoresis (PAGE) procedures to compare the mobilities of vRNAs or proteins in recombinants with those in each of their parents (*8–10*), an approach similar to that used in other

TABLE 1

METHODS FOR CLONING AND GENOTYPING INFLUENZA VIRUSES THAT WERE COLD-ADAPTED BY RECOMBINATION AT 25° WITH MUTANT A/ANN ARBOR/6/60(H2N2)

	wt parent		Recombinants		Method for identifying genes[c]							
Expt	Strain	Passage history[a]	Cloning method[b]	No. studied	RNA 1	RNA 2	RNA 3	HA	NA	NP	M	NS
CR 6[d]	A/Queensland/42/72(H3N2)	E5	1	1	R1	R1	R1	HI	NI	R1	R2	R1
CR 12[e]	A/Ann Arbor/9/73(H3N2)	E3	1	1	R1	R1	R1	HI	NI	R1	P	R1
CR 13[e]	A/Dunedin/4/73(H3N2)	E4	1	2	R2	R1	R1	HI	NI	R1	P	R1
CR 18[d]	A/Scotland/840/74(H3N2)	E4	1	5	R1	R1	R1	HI	NI	R1	R2	R1
CR 19[e]	A/Victoria/3/75(H3N2)	E4	1	1	R3	R1	R1	HI	NI	R1	R1	R1
CR 22[e]	A/Victoria/3/75(H3N2)	SPF E2	1	2	R3	R1	R1	HI	NI	R1	R1	R1
CR 29	A/Alaska/6/77(H3N2)	SPF E1,BK4,SPF E1	1	2	R1	R3	R3	HI	NI	R1	R1	R1
CR 31	A/Alaska/6/77(H3N2)	SPF E1,BK4,SPF E1	2	10	R1	R3	R3	HI	NI	R1	R1	R1
CR 33	A/USSR/90/77(H1N1)	E7[f]	1	2	R1	R1	R2	HI	NI	R2	R1	R1
CR 35	A/Hong Kong/123/77(H1N1)	SPF E1,BK5,SPF E1	1	1	R1	R1	R2	HI	NI	R2	R1	R1
CR 36	A/Hong Kong/123/77(H1N1)	SPF E1,BK5,SPF E1	2	8	R1	R1	R2	HI	NI	R2	R1	R1

[a] E indicates egg; SPF E, specific pathogen free egg; BK, bovine kidney. Viruses with BK passage had been cloned by two plaque isolations, once at 34° and then at 39°. BK passages were done by D. Brian Murphy.

[b] See text for description of methods.

[c] R1, R2, and R3 indicates polyacrylamide gel electrophoresis of virion RNA in, respectively: the absence of urea at 39 ± 1°; the absence of urea at 34 ± 1°; and in the presence of urea at 29 ± 1° (*10*). HI and NI indicate hemagglutination-inhibition and neuraminidase-inhibition tests. P, polyacrylamide gel electrophoresis of virion polypeptides (*8*).

[d] Genotyping of M and NS previously described (*8*).

[e] Full genotyping previously described (*10*).

[f] Includes two terminal dilution passages.

TABLE 2

GENE COMBINATIONS OBSERVED IN 35 VIRUSES THAT WERE COLD-ADAPTED BY RECOMBINATION WITH MUTANT A/ANN ARBOR/6/60

Combination[b]	Derivation of non-HA, non-NA, genes[a]						No. detected	
	RNA 1	RNA 2	RNA 3	NP	M	NS	With wt NA	Total
1	X[c]	X	X	X	X	X	18	23
2	X	X	X	X	X	O	5	7
3	X	O	X	X	X	X	4	4
4	X	X	X	X	O	X	1	1

[a] Viruses were selected for growth in the presence of antisera to A/Ann Arbor/6/60, and all contained HA and NA of wt virus with the exception of AA-CR 18 clone 4 and 5, -CR 22 clone 17, -CR 31 clone 4, 16, and 17, and -CR 35 clone 4 which contained NA of the A/Ann Arbor/6/60 parent.

[b] Viruses with gene combination 1 were: AA-CR 6; -CR 12; -CR 22 clone 17; -CR 29 clone 2 and 17; AA-CR 31 clones 2, 4, 13, 16, 17, 19, and 20; -CR 33 clones 1 and 2; -CR 35 clone 2; -CR 36 clones 1, 2, 3, 4, 5, 7, 9, and 10. Viruses with gene combination 2 were AA-CR 18 clones 0, 4, 5, 6, and 7; AA-CR 31 clones 10 and 12; viruses with gene combination 3 were AA-CR 13 clones 5 and 9; AA-CR 19; AA-CR 22 clone 1. Virus with gene combination 4 was AA-CR 31 clone 3.

[c] X indicates gene derived from mutant parent, O indicates gene derived from wild-type parent.

studies of influenza recombinants prepared from known parents (*11*). RNA of all recombinants except AA-CR 12 was examined under at least two conditions of electrophoresis, differing either in the presence or absence of urea as denaturant, or in the temperature of electrophoresis (Table 1). Such procedures were necessary because often one electrophoretic condition failed to produce migrational differences in some RNAs of different strains (*10*). Comparison of RNAs under different conditions also can prevent false identification of gene derivations due to aberrant RNA migration rates caused by spontaneous mutations occurring during recombination (unpublished results).

Results of gene analysis (Table 2) confirmed that each clone derived from the mixed infections was a recombinant, rather than an isolate of a ca subpopulation or spontaneous ca mutant derived from the wt viruses used. Furthermore, analysis of the much larger number of recombinants than previously studied confirmed that their gene composition was highly restricted, and only 4 of 64 theoretical combinations of non-HA, non-NA genes were detected. Virus with gene combination 4 (i.e., containing wt M protein) had not previously been identified, but the H3N2 recombinant AA-CR 31 clone 3 having this composition was ts, as were clones with gene compositions 1 and 2 derived from the same experiment (Table 3). Virus with gene composition 3 has previously been demonstrated to have a ts phenotype (*10*). Recombinants of the H1N1 virus A/Hong Kong/123/77 (e.g., CR 35 clone 2) also were ca and ts, providing additional evidence that these biological properties of the A/Ann Arbor/6/60 mutant can be transferred to viruses of different antigenic subtypes.

Fifty-one percent (18 of 35) of the ca recombinants we have now studied derive all non-HA, non-NA genes from the ca parent A/Ann Arbor/6/60, and only HA and NA from their wt parent. One explanation for this finding might be that the non-HA, non-NA genes of A/Ann Arbor/6/60 virus are highly interdependent in their replication at any temperature.

Alternatively, the consistent linkage of RNA 1, 3, and the NP gene during recombination at 25° might come about because the cluster of all three genes is essential for replication at 25°. A further possibility, perhaps the most likely one, is that genes from the A/Ann Arbor/6/60 mutant parent may predominate in recom-

binants simply because of the greater efficiency of replication of the mutant parent than of wt viruses in cells mixedly infected at 25°. We cannot at present choose between these alternative explanations for our findings. Nor can we conclude that lesions in RNAs 2, 7, and/or 8 did not originally contribute to the ca or ts properties of the A/Ann Arbor mutant because in other studies of the genetic basis for biological properties of influenza recombinants occasional exceptions have been observed that did not fit an otherwise consistent pattern (*12–14*). Thus, the possibility must be considered that some wt virus genes might undergo spontaneous mutations so that they function satisfactorily at 25°, at least when combined with genes from a ca mutant such as A/Ann Arbor/6/60.

Prior to our genotyping studies it was not known whether only a few genes of the wt virus might be replaced by genes from the ca parent when ca recombinants were produced. That situation might not result in the ca recombinants having a reproducible level of attenuation because studies with avian influenza have shown that virulence can be affected by the constellation of genes in recombinants and by the strain of avirulent virus donating genes that are substituted into a virulent virus (*7*). Results with several other candidate live influenza virus vaccines suggest the same phenomenon may apply to human influenza viruses (*5, 6*). We have now demonstrated, however, that when several different wt viruses were recombined with the ca mutant A/Ann Arbor/6/60 at 25° about half of the ca recombinants derived only the HA and NA from wt virus, but all other genes from the mutant parent. Although it has been reported that the cutoff temperature of ca recombinants may vary slightly (*15*) this has not been shown to relate to gene composition (*16*). Recent clinical studies with ca recombinants having contemporary H3N2 or H1N1 antigens have also shown that, in contrast to an earlier suggestion (*17*), attenuation for man of ca recombinants appears to be independent of cutoff temperature (*16*). Therefore, the finding that recombination with the ca parent at 25° can produce candidate live vaccine strains which have a constant gene constellation except for their HA and NA, supports the possibility that ca recombinants can be produced

TABLE 3

PLAQUE TITRATIONS IN PRIMARY CHICK KIDNEY CELLS OF RECOMBINANT INFLUENZA A VIRUSES WITH DIFFERENT GENETIC COMPOSITIONS

Viruses	Gene combination	Infectivity titer at (°)[a]		
		25	33	39
Parental				
mutant A/Ann Arbor/6/60(H2N2)	N/A	8.0	8.7	NP[b]
wt A/Alaska/6/77(H3N2)[c]	N/A	NP	7.9	6.9
wt A/Hong Kong/123/77(H1N1)[d]	N/A	NP	7.9	7.3
Recombinant				
AA-CR 31 clone 2 (H3N2)	1[e]	6.0	7.3	NP
AA-CR 31 clone 3 (H3N2)	4	5.9	6.8	NP
AA-CR 31 clone 12 (H3N2)	2	6.8	8.0	NP
AA-CR 35 clone 2 (H1N1)	1	6.3	7.7	NP

[a] Titer expressed as log PFU/ml.
[b] No plaques at 10^{-3}.
[c] wt parent of AA-CR 31 recombinants.
[d] wt parent of AA-CR 35 recombinants.
[e] See Table 2 for gene combinations.

in different years which will have similar levels of attenuation. It is hoped that this will facilitate the selection of recombinants useful for the evaluation of live influenza vaccines in the prophylaxis of influenza.

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