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Recombinant Cold-Adapted Attenuated Influenza A Vaccines for Use in Children: Molecular Genetic Analysis of the Cold-Adapted Donor and Recombinants

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A previously described cold-adapted attenuated virus, A/Leningrad/134/17/57(H2N2), was further modified by 30 additional passages in chicken embryos at 25°C. This virus had a distinct temperature-sensitive (ts) phenotype, grew well in chicken embryos at 25°C, and failed to recombine with reference ts mutants of fowl plague virus containing ts lesions in five genes coding for non-glycosylated proteins (genes 1, 2, 5, 7, and 8). Recombination of A/Leningrad/134/47/57 with wild-type influenza virus strains A/Leningrad/322/79(H1N1) and A/Bangkok/1/79(H3N2) yielded ts recombinants 47/25/1(H1N1) and 47/7/2 (H3N2). These recombinants inherited their ts phenotype and ability to reproduce in chicken embryos at 25°C from the cold-adapted parent. Analysis of the genome composition of the recombinants obtained by recombination of the cold-adapted donor with wild-type influenza virus strains A/Leningrad/322/79(H1N1) and A/Bangkok/1/79(H3N2) showed that recombinants 47/25/1(H1N1) and 47/7/2(H3N2) inherited five and six genes, respectively, from the cold-adapted parent, and hemagglutinin and neuraminidase genes from the wild-type strains.

Previously we obtained a cold-adapted attenuated influenza A virus strain, A/Leningrad/134/17/57(H2N2), containing temperature-sensitive (ts) mutations in three genes (4). Recombinant, cold-adapted, live attenuated influenza vaccines (H3N2 and H1N1) were prepared and evaluated in adults. The recombinant vaccine strains were characterized by low reactogenicity and high antigenic activity (1, 3, 4, 6).

In the present study we attempted to obtain a stable, attenuated, cold-adapted vaccine suitable for use in children 3 to 15 years old by (i) further passaging the A/Leningrad/134/17/57 strain until a majority of the genes which code for non-glycosylated proteins might have ts mutations; (ii) preparing recombinants of this donor with wild-type influenza virus strains of H1N1 and H3N2 serotypes and demonstrating that the recombinants inherited the majority of the genes from the attenuated donor, but that the hemagglutinin and neuraminidase genes were from the wild-type strains; and (iii) determining reactogenicity and antigenic activity of these recombinant vaccines in children 3 to 15 years of age.

This paper presents data on obtaining a cold-adapted donor strain and cold-adapted recombinants, on determination of genes of the donor strain which carry ts mutations, and on analysis of the genome composition of the recombinants obtained.

MATERIALS AND METHODS

Viruses. The cold-adapted A/Leningrad/134/17/57(H2N2) strain (1, 4, 6) was passaged 30 additional times in chicken embryos at 25°C. Thus, the donor strain had had a total of 47 passages in chicken embryos at 25°C and was designated A/Leningrad/134/47/57(H2N2). Influenza virus wild-type strains were A/Leningrad/322/79(H1N1), which is antigenically identical to A/Brazil/11/78/(H1N1) and had two passages in chicken embryos at 32°C, and A/Bangkok/1/79(H3N2), which had three passages at 32°C after isolation. Wild-type strain A/Bangkok/1/79(H3N2) had been cloned in chicken embryos at 40°C three times and wild-type strain A/Leningrad/322/79(H1N1) had been cloned one time, using a limiting dilutions technique before the recombination experiments to obtain virus lines with a distinct ts^+ phenotype. Studies of these lines have shown that they grow well at 40°C (Table 1), and in recombination experiments they recombine with all fowl plague virus (FPV) ts mutants used (T. E. Medvedeva, K. V. Lisovskaya, F. I. Polezhaev, L. M. Garmashova, G. I. Alexandrova, and Y. Z. Ghendon, Acta Virol., in press).

Preparations of recombinants. To obtain a recombinant with hemagglutinin and neuraminidase genes from A/Bangkok/1/79(H3N2) virus, this strain was pre-inactivated at 40°C for 24 h to the complete loss of infectivity. Chicken embryos were inoculated with a mixture of the inactivated wild-type strain and native cold-adapted A/Leningrad/134/47/57 virus ($10^{7.5}$ 50% egg infective dose [EID_{50}]) and incubated at 32°C for 48 h. The viral material obtained was cloned three times in chicken embryos at 32°C in the presence of antiserum to donor strain A/Leningrad/134/47/57 by using a limiting dilutions technique. Detailed conditions for obtaining the recombinants were described elsewhere (7–9). The recombinant selected for further studies was designated 47/7/2(H3N2). To obtain an H1N1 recombinant, the cold-adapted donor was crossed with A/Leningrad/322/79(H1N1) (antigenic analog of A/Brazil/11/78). Chicken embryos were coinfected with native viruses ($10^{7.5}$ EID_{50}) and incubated at 32°C for 18 h. The material obtained was passaged twice in the presence of antiserum to the cold-adapted donor strain and then cloned once by a limiting dilutions technique in chicken embryos at 25°C (incubation was for 72 h) in the presence of antiserum

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TABLE 1. ts phenotype of parent strains and recombinants

Strain	Reproduction in chicken embryos (log EID_{50}/0.2 ml) at:[a]		
	25°C	32°C	40°C
A/Leningrad/322/79 (H1N1), wild-type parent	1.5	6.5	5.0
A/Bangkok/1/79(H3N2), wild-type parent	1.75	8.25	6.25
A/Leningrad/134/47/57 (H2N2), cold-adapted parent	7.0	9.0	1.5
47/25/1(H1N1), recombinant	6.0	8.25	1.25
47/7/2(H3N2), recombinant	6.0	7.0	1.5

[a] The virus grown in chicken embryos at 32°C was simultaneously titrated in chicken embryos at the indicated temperatures.

to the donor strain A/Leningrad/134/47/57. The selected recombinant was designated 47/25/1(H1N1).

Recombination test. The analysis of the attenuated donor strain and the recombinants was carried out by recombination test. This was done in chicken embryo fibroblast (CEF) cultures or, in some experiments, in the MDCK cell line, using reference ts mutants of FPV belonging to different recombination groups in which the genes carrying a ts mutation were previously determined. The viruses and method used in these experiments were described earlier (4).

Determination of the genome composition of recombinants. The genome composition of the recombinants was analyzed by hybridization of labeled viral cRNA with unlabeled virion RNA, followed by treatment of double-stranded complexes with nuclease S1 and polyacrylamide gel electrophoresis (4, 5).

RESULTS

Examination of ts phenotype. Wild-type parent strains grew well at 40°C, but failed to reproduce at 25°C (Table 1).

TABLE 2. Recombination of the cold-adapted influenza virus A/Leningrad/134/47/57 with FPV ts mutants in CEF culture[a]

ts mutant	Mutant gene	Mutant protein	Log PFU/ml in CEF cultures infected with: FPV ts mutants and human influenza virus: A/Leningrad/134/47/57		A/Krasnodar/101/59 (control)		FPV ts mutants only	
			36°C	42 or 40°C	36°C	42 or 40°C	36°C	42 or 40°C
29	1	PB2	7.5	<2	7.0	6.5	7.3	<2
131	2	PB1	5.3	<2	5.6	4.0	5.0	<2
166	3	PA	6.7	5.0	6.5	5.0	6.5	<2
US1	5	NP	6.0	<2	6.3	5.5	6.0	<2
303/1	7	M	5.8	<2	5.8	3.8	5.7	<2
mN3	8	NS	6.0	<2	5.9	4.5	6.3	<2

[a] CEF cultures were infected with the cold-adapted A/Leningrad/134/47/57(H2N2) strain and with the wild-type A/Krasnodar/101/59(H2N2) strain as a control at a dose of 1 to 2 EID_{50} per cell. After 30 min of adsorption at 20°C, the cells were infected with FPV ts mutants at a dose of 10^1 to 10^6 PFU per culture. After 30 min of adsorption, the cells were supplemented with agar and incubated at the optimal (36°C) and nonpermissive temperature (42°C for ts mutants 29, 131, 166, and 303/1; 40°C for US1 and mN3) for 72 h after which plaques were counted. Human influenza viruses failed to form plaques in CEF cultures under the conditions used.

TABLE 3. Recombination of the cold-adapted influenza virus A/Leningrad/134/47/57 with FPV ts mutants in MDCK cells[a]

ts mutans	Mutant gene	Virus titer (PFU/ml) FPV ts mutants × A/Leningrad/134/47/57		FPV ts mutants only	
		36°C	40 or 42°C	36°C	40 or 42°C
29	1	3.1×10^6	<2	2.2×10^6	<2
131	2	2.1×10^6	<2	3.4×10^6	<2
166	3	4.8×10^6	1.1×10^5	5.2×10^6	<2
US1	5	8.7×10^5	<2	6.6×10^5	<2
303/1	7	9.1×10^5	<2	8.3×10^5	<2
mN3	8	1.6×10^6	<2	3.7×10^6	<2

[a] MDCK cultures were infected with viruses (1 EID_{50} per cell of each partner or 2 EID_{50} per cell on self-crossing) and incubated at 36°C for 18 h in the presence of 4 μg of trypsin per ml. After that, virus titers were determined in CEF cultures by using a plaque technique at 36°C and at the nonpermissive temperature (40°C for US1, 303/1, and mN3; 42°C for ts 29, 131, and 166).

At the same time, the cold-adapted parent and both recombinants possessed a distinct ts_{40}^- phenotype but were able to grow well at 25°C.

The cold-adapted A/Leningrad/134/47/57 strain, which had had 47 passages in chicken embryos at 25°C, did not recombine in CEF culture with FPV mutants ts29, ts131, tsUS1, ts303/1, and tsmN3, which have ts lesions in genes 1, 2, 5, 7, and 8, respectively (Table 2). Therefore, it is likely that five of six nonglycosylated viral proteins (PB1, PB2, NP, M, and NS) of A/Leningrad/134/47/57 contain ts mutations.

Since there are data in the literature concerning dependence of manifestation of the ts phenotype of influenza virus on the host-cell (td-hr mutants) (10), we performed a recombination analysis of cold-adapted donor with FPV ts mutants in mammalian cell line MDCK (dog kidney cells). The data obtained turned out to be identical to those obtained in CEF culture (Table 3). It should be noted that all ts mutants of the FPV Weybridge strain (H7N7) reproduced and formed plaques in MDCK culture.

Genome composition of the recombinants obtained between the cold-adapted donor and actual wild-type influenza virus strains. Recombinant 47/25/1(H1N1) was shown by RNA hybridization and nuclease digestion to inherit genes 2, 3, 5, 7, and 8 from the cold-adapted parent, but gene 1 and two genes coding for the hemagglutinin and neuraminidase glycoproteins were from the wild-type strain (Fig. 1 and Table 4). Recombinant 47/7/2(H3N2) was similarly shown to inherit all the genes coding for nonglycosylated proteins (1, 2, 3, 5, 7, and 8) from the cold-adapted parent, and only genes 4 and 6, which code for hemagglutinin and neuraminidase, respectively, were from the wild-type strain (Fig. 1 and Table 4).

Genetic analysis of the ts phenotype of the recombinants. In a recombination analysis with reference FPV mutants, recombinant 47/7/2 failed to recombine with mutants with lesions in genes 1, 2, 5, 7, and 8, whereas recombinant 47/25/1 failed to recombine with mutants with lesions in genes 2, 5, 7, and 8 (Table 5). In addition, recombinant 47/25/1 failed to recombine with mutants with a lesion in gene 3. Thus, the results with recombinant 47/7/2 were consistent with the analysis of its gene derivation as determined by RNA hybridization. Recombinant 47/25/1, however, differed from its parental cold-adapted strain in that it failed to recombine with a ts mutant with a lesion in RNA 3. Apparently, this might have resulted from three additional passages at 25°C, which were carried out in the process of obtaining and

selecting recombinant 47/25/1. It should be noted that parent strain A/Leningrad/322/79(H1N1) in the same recombination test formed ts^+ recombinants with all six FPV ts mutants (not shown).

DISCUSSION

Lately, cold-adapted, attenuated, recombinant live influenza vaccines that are areactogenic and have a high antigenic activity for immunization of adults have been developed in the USSR and the United States. The data on development and evaluation of such vaccines have been recently reviewed in a joint paper by Soviet and American scientists (6).

The cold-adapted donor strain used in the USSR for producing recombinant cold-adapted vaccine for adults (A/Leningrad/134/17/57) is proposed to have ts mutations in three genes (4). Recombinants of this donor and various wild-type influenza A viruses which have been circulating after 1972 proved to be safe for persons older than 16, since they caused no vaccination reactions even after intranasal immunization of doubly seronegative individuals who lacked both anti-hemagglutinin and anti-neuraminidase antibody in their serum (4, 6, 7). However, since children, particularly of preschool age, are highly susceptible to influenza illness, we decided to prepare a special donor of attenuation which could be used to construct recombinant live vaccine strains for children. After 30 additional passages of the cold-adapted A/Leningrad/134/17/57 strain at 25°C, we obtained the A/Leningrad/134/47/57 variant. Analysis of this variant in chicken embryos at 25°C in a test of recombination, in CEF culture, or in the MDCK cell line with reference FPV ts mutants with known genes carrying mutation lesions showed that the variant obtained fails to recombine with ts mutants with mutations in genes 1, 2, 5, 7, and 8. These data suggest that this variant has mutations in the genes coding for nonglycosylated proteins (PB1, PB2, NP, M, and NS). Taking into account, however, that the analysis of data obtained by a recombination test may be complicated by such phenomenum as extragenic suppression or intragenic complementation, definitive proof of the site of ts mutations will require further detailed genetic and molecular analysis.

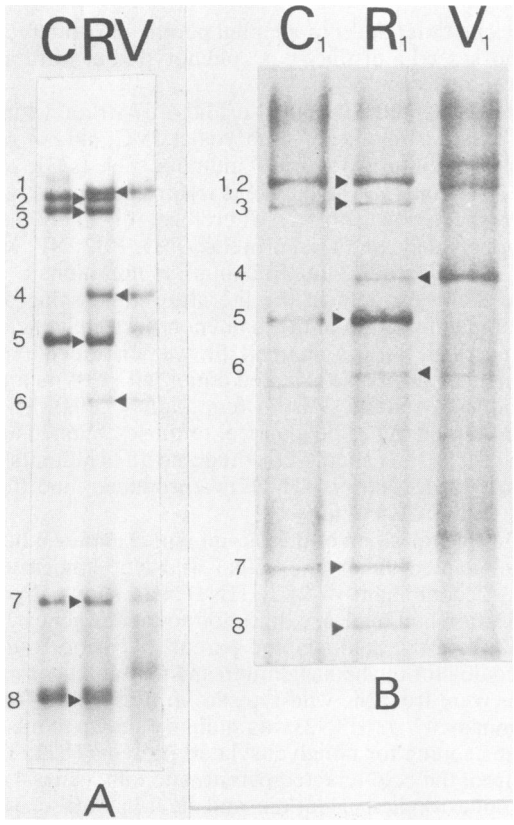

FIG. 1. Analysis of the genome composition of the recombinants. Cells infected with the recombinants and parent viruses (100 EID_{50}/ml) were incubated in the presence of cycloheximide (100 μg/ml) and [H^3]uridine at 36°C for 4 h. [H^3]RNA (cRNA) was then extracted and hybridized with excess unlabeled virion RNA isolated from purified virions of the recombinants. The material was treated with nuclease S1 and analyzed by electrophoresis in a 4% polyacrylamide gel. (A) Analysis of recombinant 47/25/1(H1N1). Lane C, cold-adapted parent, A/Leningrad/134/47/57; V, wild-type strain A/Leningrad/322/79(H1N1); R, recombinant 47/25/1. (B) Analysis of recombinant 47/7/2(H3N2). Lane C_1, cold-adapted parent, A/Leningrad/134/47/57; V_1, wild-type strain A/Bangkok/1/79(H3N2); R_1, recombinant 47/7/2. Numbers 1 through 8 to the left of the gels correspond to double-stranded RNAs. The arrows are between double-stranded RNA complexes with similar electrophoretic mobilities.

TABLE 4. Genome composition of the recombinants obtained by crossing the cold-adapted and wild-type influenza virus strains[a]

Parents	Recombinant	Distribution of gene:[b]							
		1	2	3	4	5	6	7	8
A/Leningrad/134/47/57(H2N2) (cold-adapted) × A/Leningrad/322/79(H1N1) (wild-type)	47/25/1(H1N1)	W	C	C	W	C	W	C	C
A/Leningrad/134/47/57(H2N2) (cold-adapted) × A/Bangkok/1/79(H3N2) (wild-type)	47/7/2(H3N2)	C	C	C	W	C	W	C	C

[a] CEF cultures were incubated for 30 min at 36°C in the presence of cycloheximide (100 μg/ml), and then the cells were infected with the recombinants and parent strains (100 EID_{50} per cell) and incubated for 60 min at 36°C in medium containing cycloheximide (100 μg/ml). [H^3]uridine was then added, and the incubation was continued for 3 h in the presence of cycloheximide. RNA was extracted according to the method of Hay et al. (5) and hybridized with an excess of unlabeled virion RNA isolated from purified virions of parent virus strains. The material was treated with nuclease S1, precipitated with ethanol, and analyzed by electrophoresis in a 4% polyacrylamide gel.

[b] C, a gene derived from the cold-adapted parent; W, a gene derived from the wild-type strain.

TABLE 5. Recombination of the cold-adapted recombinants with FPV ts mutants[a]

ts mutant	Mutant gene	Log PFU/ml in CEF cultures infected with:							
		PFV ts mutants and:				A/Krasnodar/101/59 (control)		FPV ts mutants only	
		47/25/1		47/7/2					
		36°C	42 or 40°C	36°C	42 or 40°C	36°C	42 or 40°C	36°C	42 or 40°C
ts29	1	7.5	4.7	7.3	<2	7.3	6.5	7.3	<2
ts131	2	8.0	<2	7.5	<2	7.9	6.6	8.0	<2
ts166	3	7.0	<2	7.3	5.0	7.0	6.0	7.3	<2
US1	5	7.3	<2	7.2	<2	7.3	6.0	7.5	<2
303/1	7	7.0	<2	7.1	<2	7.3	6.0	7.3	<2
mN3	8	6.3	<2	6.1	<2	6.3	5.3	6.0	<2

[a] See Table 2, footnote *a*.

The analysis of the genome composition of the recombinants obtained by recombination of the cold-adapted donor with wild-type influenza virus strains A/Bangkok/1/79(H3N2) and A/Leningrad/322/79(H1N1) showed that recombinant 47/7/2(H3N2) inherited all six genes coding for non-glycosylated proteins from the cold-adapted donor and the genes coding for hemagglutinin and neuraminidase from the wild-type strain; recombinant 47/25/1(H1N1) inherited five genes from the cold-adapted parent, whereas gene 1 and the genes coding for the hemagglutinin and neuraminidase were derived from the wild-type strain. Both recombinants retained a ts phenotype identical to that of the cold-adapted donor strain.

As shown in a companion paper (2), the recombinants obtained proved to be weakly reactogenic for children of 3 to 15 years of age but possessed high antigenic activity and genetic stability.

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