Acta virol. **35**: 332-342, 1991

FURTHER STUDIES ON COLD ADAPTED VARIANTS OF HUMAN INFLUENZA VIRUS A/KRASNODAR/101/59 (H2N2) WITH SPECIAL REFERENCE TO GENETIC STABILITY OF ATTENUATION MARKERS

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Received April 3, 1989; revised September 24, 1990

Summary. - A previously described cold adapted (ca) attenuated virus, K/25, derived from parent strain A/Krasnodar/101/59 (H2N2), was further modified by 35 additional passages in chick embryos at suboptimal temperature. The virus obtained had retained a distinct ts and ca phenotype and some other markers of attenuation but differed from formerly isolated ca variants by its higher genetic stability connected with an increased growth capacity in chick emryos.

Key words: influenza virus; cold adapted attenuated strain; ts mutation

Introduction

In a previous paper (Panzig *et al.*, 1984) we reported on characterization of some ts mutants isolated in the course of cold passages. Despite of the fact that ts mutations could be detected in 5 or 6 genes respectively, the genetic stability after 25 and/or 40 cold passages was not sufficient. Furthermore, it could be shown that growth capacity in chick embryos decreased in the course of passages. In order to obtain more stable mutants and to increase simultaneously the growth capacity in chick embryo, we decided to choose the 25th cold passage utilizing its good attenuation as new starting point for further cold passages.

Materials and Methods

Obtaining of the variants. Human influenza virus A/Krasnodar/101/59 strain was passaged in CEF cultures in the presence of trypsin as previously described (Panzig *et al.*, 1984). After 25 passages at 28 ^{0}C in CEF cultures the passages were continued in the chick embryo at 27 ^{0}C (Fig. 1).

Determination of attenuation markers. Testing of ca phenotype was done in CEF cultures in the presence of trypsin by comparing the infectivity titre at 27 ^{0}C and 33 ^{0}C (PFU/ml). The ts phenotype was determined by titration in chick embryos (CE) at 33 ^{0}C and 39.5 ^{0}C.

Studies on virus reproduction in the lungs of hamsters were performed according to the method of Spring *et al.* (1975, 1977) as described in detail earlier (Panzig *et al.*, 1984).

Recombination test for detection of ts mutations. Recombinants were prepared in CEF cultures using ts mutants of FPV with ts mutations in genes 1, 2, 3, 5, 6, 7 and 8 according to the method of Ghenkina and Ghendon (1979) as previously described (Lisovskaya *et al.*, 1981; Ghendon *et al.*, 1984). For identification of the ts mutation in gene 4, recombination was performed in chick embryos (CE) as described elsewhere (Panzig *et al.*, 1984).

Genome analysis of the ca variant. The genome composition of the variant was analyzed according to the method of Hay *et al.* (1977). Moreover, a separation of single stranded (ss) RNA was done by electrophoresis in 3 % acrylamide gel containing 8 mol/l urea according to the method of Floyd *et al.* (1974).

Determination of genetic stability of ca variants in chick embryos. Seven sucessive passages in chick embryos at 37 ^{0}C with subcultivation at 39.5 ^{0}C between the passages were performed. Titrations were done in CE at 33 ^{0}C. In some different way virus was pasaged alternatively at 39.5 ^{0}C and 33 ^{0}C in CE altogether 7 times. Another test was done by successive passages at semipermissive temperature (37 ^{0}C) using high inoculation doses.

Genetic stability of the ca variants in animals was studied by determination of the ca and ts phenotypes of given virus grown in the lungs of hamsters by comparing the plating efficiency at 27 ^{0}C, 33 ^{0}C, and 39.5 ^{0}C respectively, and additionally by inoculation of CE and subsequent incubation at 39.5 ^{0}C followed by titration at 33 ^{0}C.

Evaluation of antigenic and immunogenic activity of the ca variants in animals. Paired sera were taken from hamsters before vaccination and 21 days after infection by the intranasal route with 10^5 ID_{50}. Antibody rise was measured by means of haemagglutination inhibition (HI) test. After 3 weeks each animal was challenged with 10^5 ID_{50} of the egg-grown virulent strain A/Krasnodar/101/59.

Studies on non-transmissibility of the ca variant in animals. Two out of 10 hamsters were infected with $10^5 ID_{50}$ by the intranasal route. After 1 hr separation, the infected animals were put together with the non-infected ones. Blood samples taken from hamsters before infection and 21 days post infection were tested by means of HI test and compared with sera from non-infected hamsters.

Haemagglutination inhibition test. The test was performed as micromethod according to the recommendation of WHO (1982). Sera were treated with KJO_4 and then incubated at 56 ^{0}C for 30 min to destroy the non-specific inhibitors.

Preparation of ca reassortant viruses with actual surface glycoproteins. Allantoic fluid of wild type strain A/Dresden/1/83, diluted 1:10, was inactivated by UV-light. Samples with reduced infectious titre of 10^3, 10^4, and 10^5 EID_{50} were diluted to an endpoint of 10^{-1} EID_{50}/ml, mixed with equal parts of ca variant K/60 (at each case 10^3 EID_{50}) and inoculated into embryonated eggs. The allantoic fluid harvested after 48 hr was treated with antisera against A/Krasnodar/101/59 (HI-titre 1:5120) for eliminating viruses containing haemagglutinin and neuraminidase of the ca variant. This procedure was repeated 3 times-followed by 2 - 4 plaque passages in CEF cultures.

Results

Biological properties of the ca variant K/60

The results shown in Table 1 demonstrate that the ca variant K/60 retained the distinct ts and ca phenotypes of the former ca variant K/25. Some other markers of attenuation as plaque size, decreased stability of infectivity after 30 min incubation at 50 ^{0}C, and reduced capacity of reproduction in lungs of hamsters also could be detected. But in contrary to the ca variant K/25 and to the parent strain A/Krasnodar/101/59, the new variant had an increased growth capacity in chick embryos.

Identification of genes containing ts mutations

The data presented in Table 2 illustrate that by crossing the variant K/60 with

Table 1. Biological properties of cold adapted variant K/60 in comparison to the parent strain A/Krasnodar/101/59 and to the strain of the 25th cold passage, K/25

	ca*)		ts**)		Plaque ø	Stability	Virulence
	27 °C	33 °C	33 °C	39.5 °C	33 °C mm 4 days post infection	of infectivity logN - logNo 30' 50 °C	in hamster, log EID_{50}/g of lungs
A/Krasnodar/101/59	< 1.0	7.48	8.15	7.0	2.5	1.78	5.98 ± 0.4
K/25	7.18	7.7	8.3	< 3.0	5 - 6	3.04	3.28 ± 0.74
K/60	7.0	7.69	9.49	< 1.0	4 - 5	3.37	2.77 ± 0.95

*) Titres in CEF cultures (log_{10} PFU/ml) in the presence of trypsin
**) Titre in log_{10} EID_{50}/ml

Table 2. Detection of ts mutations of cold adapted variants in single genes by recombination with FPV ts-mutants in CEF at different temperatures*

Ts mutants	Mutant genes	Mutant proteins	Plaque formation in CEF cultures infected with FPV ts mutants and variants of human influenza virus in the absence of trypsin							
			K/25		K/60		A/Krasn./101/59		FPV ts mutants only	
			36 ^{0}	40 ^{0} or 42 ^{0}	36 ^{0}	40 ^{0} or 42 ^{0}	36 ^{0}	40 ^{0} or 42 ^{0}	36 ^{0}	40 ^{0} or 42 ^{0}
29	1	PB2	1×10^8	<2	2×10^8	1×10^7	2×10^8	1×10^7	2×10^8	<2
131	2	PB1	9×10^7	7×10^5	1×10^8	6×10^5	1×10^8	1×10^6	1×10^8	<2
166	3	PA	2×10^7	<2	7×10^6	<2	8×10^6	7×10^5	8×10^7	<2
US1	5	NP	5×10^6	<2	1×10^7	<2	1×10^7	6×10^5	1×10^7	<2
5	6	NA	1×10^7	<2	1×10^8	<2	1×10^8	3×10^6	1×10^8	<2
303/I	7	M	4×10^6	5×10^5	2×10^6	1×10^5	3×10^6	1×10^5	2×10^6	<2
mN3	8	NS	1×10^7	2×10^5	1×10^7	2×10^5	2×10^7	3×10^5	1×10^7	<2

*) For identification of ts mutation in gene 4 the recombination was performed in CE at 33 ^{0}C. Ts phenotype of the virus population was determined by titration after incubation at 40 ^{0}C and 33 ^{0}C, respectively.

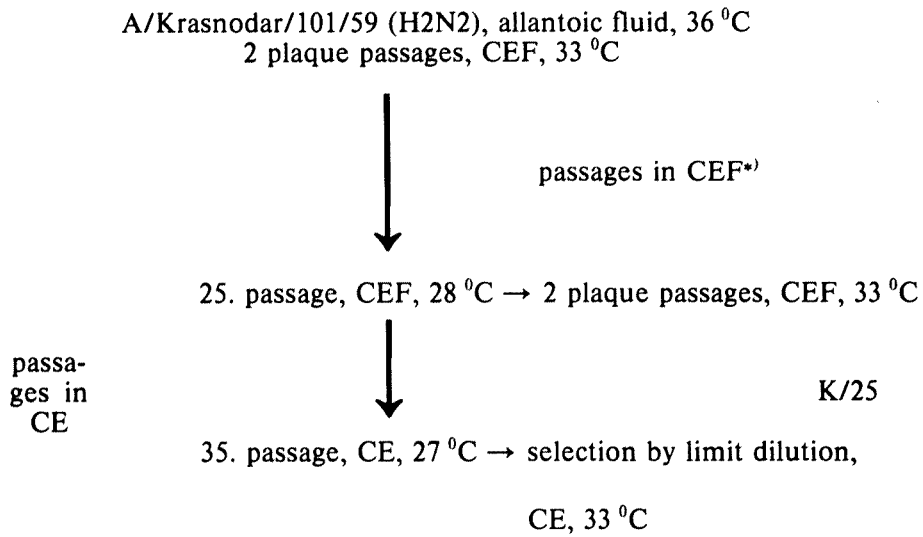

Fig. 1
The passage history of the cold adapted variant K/60 passaged in CE fibroblasts (CEF) in the presence of trypsin

FPV ts mutants ts 29, ts 131, ts 303/1, ts mN3 containing ts mutations in genes 1, 2, 7, and 8, ts+ recombinants capable of inducing plaques at the non-permissive temperature were formed. By analyzing the ts phenotype of clones isolated from plaques at the non-permissive temperature it could be excluded that an intracistronic complementation did occur.

Crossing the variant K/60 with FPV ts mutants ts 166, ts US1, and ts 5, containing ts mutations in genes 3, 5, and 6 respecively, did not yield recombinants capable of forming plaques under non-permissive conditions. The results obtained allowed us to conclude that the variant K/60 contained ts mutations in genes 3, 5, and 6. Crossing the variant K/60 with FPV mutant ts 46 in chick embryos the manifestation of a ts mutation in gene 4 could be proved (not shown).

Studies of the genome of ca variants

Since passages of viruses at suboptimal temperature might involve the acquisition of other than ts mutations not detectable in the recombination test we decided to apply the analysis of double stranded cRNA-vRNA hybrids and of single stranded vRNAs for detection of mutations in individual genes of the ca variant. By hybridization of vRNA of the parent strain A/Krasnodar/101/59

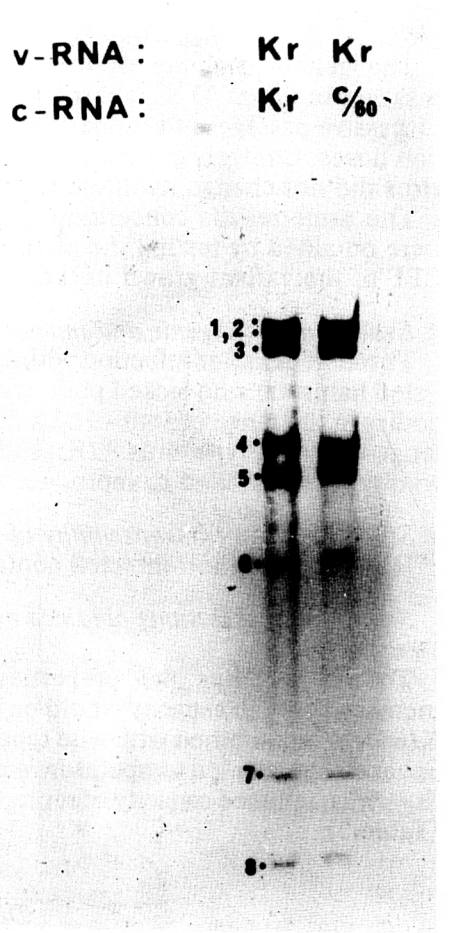

Fig. 2
Electrophoretic analysis of the mobility of ds RNA obtained after hybridization of ^{3}H-labelled cRNA from ca variants with vRNA of influenza virus strain A/Krasnodar/101/59
CEF were infected with viruses and incubated in the presence of cycloheximide and (^{3}H)-uridine for 3 hr at 36 ^{0}C, cRNA was then extracted and hybridized with an excess of unlabelled vRNA. The samples were treated with S1 nuclease and analysed by electrophoresis in a 7.5 % polyacrylamide gel containing 6 M urea. 1, 2, 3, 4, 5, 6, 7, 8 – location of corresponding double-stranded complexes of cRNA/vRNA (Kr=A/Krasnodar/101/59; C/60=ca variant).

with cRNA of the variant K/60 major differences were observed in the electrophoretic mobility of segments 4, 5, 6, and 8, coding for the proteins HA, NP, NA, and NS (Fig. 2). Using the method of ss vRNA analysis, a further difference in electrophoretic mobility of segment 7 coding for the M protein could be detected possibly due to changes in the secondary structure (not shown). The results obtained by recombination test together with results of genome analysis

allowed us to conclude that ca variant K/60 contained ts mutations in genes 3, 4, 5, and 6 and additionally mutations in genes 7 and 8 which apparently were not ts mutations because they could not be revealed in the recombination test with FPV ts mutants.

Studies of the genetic stability of the ca variant

The genetic stability of the ca variant was followed during successive passages in CE at 33 °C, alternating passages at 39.5 °C and 33 °C and during successive passages at semipermissive temperature (37 °C) with high inoculation doses. Controls at each passage in altogether 7 passages showed that the virus did not change its initial ts phenotype (Table 4).

The same results concerning the genetic stability of ts and ca phenotype were obtained by testing the plating efficiency at 27 °C, 33 °C, and 39.5 °C in CEF of the variant grown in the lungs of hamsters.

Evalution of antigenic and immunogenic activities of the ca variant

Three weeks after infection with 10^5 ID_{50} of the ca variant each of the 9 vaccinated hamsters who lacked preinoculation serum HI antibody developed antibodies in the range of 1:40 - 1:320. Moreover, it could be shown that after challenge with wild type virus A/Krasnodar/101/59, by 6 weeks post-infection the wild type virus failed to reproduce in the lungs of hamsters.

Studies on non-transmissibility of the ca variant

None of the 8 non-infected contact animals developed serum antibodies.

Studies on reassortants obtained between the ca donor and an actual wild type virus

To see whether the properties of attenuation, genetic stability, and increased growth capacity would be lost by exchange of HA and NA, the strain K/60 was recombined with wild type influenza virus A/Dresden/1/83 (H3N2). Several cold adapted temperature sensitive recombinants of high genetic stability with a reduced capacity of reproduction in lungs of hamsters were obtained (Table 3).

Discussion

Genetic stability is one of the prerequisites to an attenuated influenza virus which might serve as donor of its attenuating genes in producing recombinant live vaccines (Aleksandrova *et al.*, 1984). Furthermore, virus yields in chick embryos should be as high as possible. The aim of the present study was to stabilize the property of attenuation of a formerly prepared ca variant and to increase growth capacity in chick embryos at the same time. As can be seen from Table 2, the strain K/60 retained some desirable properties expressed already after 25 cold passages as there were several markers of attenuation and

Table 3. Biological properties of ca reassortants derived by mating ca strain K/60 with wild type strain A/Dresden/1/83 (H3N2)

		EID_{50}/0,25 ml				
		27 ^{0}C	33 ^{0}C	39.5 ^{0}C	Stability of infectivity logN – logN_0 30' 50 ^{0}C	Virulence in hamsters logEID_{50}/g of lungs
Parent strain A/Dresden/1/83		4.88	8.58	6.67	2.46	7.53±0.38
Reassortants						
K/60		7.73	8.98	<1.0	3.37	2.77±0.95
40/16	genes	n.d.	7.96	<1.0	3.38	5.3 ±0.86
40/17/1/1	derived from	7.44	8.34	<1.0	2.66	3.92±0.53
40/25	ca-variant:	6.89	8.43	<1.0	3.14	3.89±0.88
40/28/1	1, 2, 3, 5	6.84	8.73	<1.0	2.95	3.19±0.21
40/28/2	7, 8	6.80	8.16	<1.0	3.69	2.79±1.0
42/1:	genes derived from ca-parent: 4, 6, 7	n.d.	9.59	n.d.	2.75	5.33±0.62
42/8:	genes derived from ca-parent: 4, 6	6.40	8.60	6.36	2.83	6.54±0.58

Table 4. Testing of the genetic stability of ts marker of the cold adapted strain K/60 by different passage regimes in the allantoic cavity of embryonated eggs and titration

Testing regime	Titre at 33 ^{0}C log EID_{50}/0.25 ml
1. seven successive passages at 33 ^{0}C and titration of progeny at 33 ^{0}C	7.6
2. seven passages at 33 ^{0}C and from each of them a subpassage at 39.5 ^{0}C and subsequent titration of the progeny at 33 ^{0}C	<1
3. seven successive passages at 37 ^{0}C (high multiplicity) and from each of them a subpassage at 39.5 ^{0}C and subsequent titration of the progeny at 33 ^{0}C	<1
4. seven alternating passages at 33 ^{0}C and 39.5 ^{0}C and subsequently titration of the progeny of the last 39.5 ^{0}C-passage at 33 ^{0}C	1.5

decreased virulence for hamsters, but none had acquired the capacity of high yielding in chick embryos. Particular attention was paid to genetic stability of this variant. In a previous paper (Panzig *et al.,* 1984) we described the cold adapted variants also derived from human influenza virus A/Krasnodar/101/59 with ts mutations in 5 or 6 genes respectively. But despite of these multiple ts mutations a tendency of reversion to ts+ phenotype could be observed.

The new going-out for derivation of the ca variant K/60 had been the 25th cold passage of the parent strain A/Krasnodar/101/59. Surprisingly it could be demonstrated that the ts mutation in gene 1 detectable after 25 passages disappeared in the course of additional cold passages in chick embryos (Table 2). Nevertheless, the strain K/60 seemed to be a genetically more stable ca variant in comparison to formerly obtained mutants as judged by our experiments provocating the ts phenotype. The above findings suggest that there is not a well established correlation between the number of genes carrying ts mutations and genetic stability of attenuation. The results allowed us to conclude that there might be a relationship between genetic stability and high growth capacity of the strain K/60. To find out whether the attenuated strain K/60 would be a suitable donor virus we studied if it would reproducibly confer the following properties on reassortants: satisfactory level of attenuation and stability of the attenuation phenotype (ca and ts markers, virulence for hamster).

We found that 5 reassortants bearing the six genes that code for nonsurface proteins („internal genes") from the strain K/60 also were cold adapted temperature sensitive viruses of high genetic stability. Despite of identical gene composition of the reassortants 40/16, 40/17/1/1, 40/28/1, 40/28/2 (Table 3) virus yields in lungs of hamsters differed by 2.79+/- 1.0 (reassortant 40/28/2) or 5.3+/- 0.86 (reassortant 40/16). These differences possibly could result from further changes of the genome due to point mutations additionally acquired in the process of mating the parent strains. So it was obvious that the HA gene of the strain 40/28/1 must have had undergone further mutational changes since opposite to the other reassortants this strain was a large plaque, inhibitor resistant variant. Despite of relatively less depressed virus replication seen after infection of animals with ca reassortants 40/16 and 40/28/1 compared to the other reassortants with similar genome structure these strains seemed to be as stable in regard of their attenuation markers as strains mentioned above, as could be proved by altogether 5 - 7 alternating passages at 39.5 ^{0}C and 33 ^{0}C without arising of revertants. Exchange of the surface glycoproteins only did not result in distinct reduction of virulence in hamsters as can be seen from ca reassortant 42/8 bearing the genes 4 and 6 from the ca parent (Table 3).

The present study demonstrates that strain K/60 possibly could serve as donor of attenuated genes for vaccine strains.

References

Aleksandrova, G. I., Polezhaev, F. I., Budilovsky, G. N., Garmashova, L. M., Topuria, N. A., Egorov, A.Y., Romejko-Gureo, Y. R., Koval, T. A., Lisovskaya, K. V., Klimov, A. I., and Ghendon, Y. Z. (1984): Recombinant cold-adapted attenuated influenza A vaccines for use in children: Reactogenicity and antigenic activity of cold-adapted recombinants and analysis of isolates from the vaccines. *Infect. Immun.* **44**, 734–739.

Ghendon, Y. Z., Klimov, A. I., Blagoveshenskaya, C. V., and Ghenkina, D. E. (1979): Investigation of recombinants of human influenza and fowl plague viruses. *J. gen. Virol.* **43**, 183–191.

Ghendon, Y. Z., Polezhaev, F. I., Lisovskaya, K. V., Medvedeva, T.E., Aleksandrova, G. I., and Klimov, A. I. (1984): Recombinant cold-adapted attenuated influenza A vaccines for use in children: Molecular genetic analysis of the cold-adapted donor and recombinants. *Infect. Immun.* **44**, 730–733.

Ghenkina, D. B., and Ghendon, Y. Z. (1979): Recombination and complementation between orthomyxoviruses under conditions of abortive infection. *Acta virol.* **23**, 97–106.

Hay, A. J., Bellamy, A. R., Abraham, G., Skehel, J. J., Brand, C. N., and Webster, R. G. (1977): Procedures for characterization of the genetic material of candidate vaccine strains. *Develop. Biol. Stand.* **39**, 15–24.

Lisovskaya, K. V., Garmashova, L. M., and Medvedeva, T.E. (1981): Analysis of ts mutations of cold-adapted influenza A/Leningrad/134/57 virus variants. *Acta virol.* **25**, 415–417.

Panzig, B., Döhner, L., Lisovskaya, K. B., Ghendon, Y. Z. (1984): Ts mutations in the genome of cold-adapted variants of human influenza virus A/Krasnodar/101/59 (H2N2) at different passage levels and their reproduction in lungs of hamsters. *Acta virol.* **28**, 369–379.

Spring, S. B., Nusinoff, S. R., Tierney, E. L., Richman, D. D., Murphy, B. R., and Chanock, R. M. (1975): Temperature-sensitive mutants of influenza virus. III. Genetic and biological characterization of ts mutants of influenza virus A (H3N2) and their assignment to complementation groups. *Virology* **66**, 542–550.

Spring, S. B., Massab, H. P., Kendal, A. P., Murphy, B. R., and Chanock, R. M. (1977): Cold-adapted variants of influenza virus A. I. Comparison of the genetic properties of ts mutants and five cold-adapted variants of influenza virus A. *Virology* 77, 337–345.

WHO Collaborating Centers for Reference and Research on Influenza, 1982: *Concepts and Procedures for Laboratory-Based Influenza Serveillance.* U.S. Department of Health and Human Services. Centers for Disease Control, Atlanta.