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# PECULIARITIES OF OBTAINING AND CHARACTERIZATION OF A COLD-ADAPTED A/PR/8/34 INFLUENZA VIRUS VARIANT

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*Summary.* — The dynamics of alterations in biological properties of A/PR/8/34 virus strain was studied in the process of prolonged adaptation to the growth in chick embryos (CE) at a lowered (25 °C) cultivation temperature. The variant selected after 59 passages and subsequent cloning at the above temperature retained the high reproductive capacity in CE at optimal temperature (34 °C) — a characteristic of the original strain. Unlike to the latter, it showed a distinctly reduced ability to reproduce at 40 °C and a lower level of pathogenicity for white mice and CE. Analysis of genes of the cloned cold-adapted A/PR/8/34 strain detected 5 ts mutations in genes 1, 3, 5, 6 and 7 coding for P3, P2, NP, NA and M proteins, respectively.

*Key words: influenza virus; reproductivity; temperature-sensitivity; pathogenicity; gene; mutant*

## *Introduction*

The recombination test which has been successfully used both in our country and abroad to obtain vaccine strains for live influenza vaccine consists in crossing epidemic viruses with donors of attenuation, i.e. viruses with an outdated antigenic structure, which are safe for humans. Cold-adapted viruses, i.e. viruses with numerous passages at 25°—28 °C being attenuated due to the presence in their genomes of multiple ts mutations which can be transferred by recombination, proved to be the most reliable donors of attenuation (Alexandrova *et al.*, 1979; Ghendon *et al.*, 1981; Kendal *et al.*, 1982).

In a number of Western countries, the A/PR/8/34 strain which had had multiple passages in chick embryos at the optimal temperature (the so-called hr variant) has been used as the donor of attenuation. Its distinctive feature is a high reproductive capacity in CE — a very essential property for obtaining strains for killed influenza vaccine. However, high-yielding re-

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combinants obtained on the basis of A/PR/8/34 virus can retain residual reactogenicity for humans (Beare *et al.*, 1978), which makes impossible to use them for the production of live influenza vaccine without additional attenuation achieved by subsequent passages in CE in the presence of serum containing γ-inhibitors (Rytel *et al.*, 1975). In the present work we attempted to adapt the strain A/PR/8/34 to the growth at temperature of 25 °C so that the virus might acquire essential biological properties characteristic of the cold-adapted viruses and retain its initial reproductive capacity for growing CE at the optimal temperature.

## *Materials and Methods*

*Viruses.* The following strains were used: human A/PR/8/34 (H1N1) influenza virus received from WHO Center of Influenza (Atlanta, U.S.A.); cold-adapted variants of this virus which had had 30, 40, 50 and 60 passages in CE at 25 °C and were designated A/PR/8/30/25, A/PR/8/40/25, A/PR/8/50/25 and A/PR/8/60/25, respectively. The A/PR/8/59/1 clone was selected from the A/PR/8/59/25 population after a single cloning using the limiting dilutions technique in CE at 25 °C. Ts mutans of fowl plague virus (FPV) belonging to 7 recombination-complementation groups were used. Their obtaining and characteristics were described earlier (Ghendon *et al.*, 1981).

*Passages in CE at a lowered temperature.* The virus was passaged in CE at temperature of 25 °C. CE were infected with the original A/PR/8/34 virus at a dose of $10^5-10^6$ $EID_{50}$/0.2 ml and incubated for 72–96 hr. The total number of passages at 25 °C was 60. Allantoic fluids with the maximum haemagglutinating titres were used for each successive passage.

*Evaluation of the temperature-sensitivity of viruses.* Groups of 5 CE were simultaneously infected with 10 $EID_{50}$ of one of the cold-adapted variants of A/PR/8/34 influenza virus obtained at various levels of passages at 25 °C and incubated at 34 °C for 48 hr. Temperature-sensitivity of virus reproduction ($RCT_{40}$ marker) was evaluated by comparative titration of allantoic fluids from 5 CE at optimal (34 °C) and non-permissive (40 °C) temperatures. When the difference in titres was $\leq 2.0$ log $EID_{50}$, the viruses were considered to possess the $RCT_{40}$ phenotype ($RCT_{40}^+$); with a difference of greater than 5.0 log $EID_{50}$, the variants were considered for $RCT_{40}^-$, and when it was within the range of 2.0–5.0 log $EID_{50}$, the viruses were $RCT_{40}^{\pm}$.

*Pathogenicity for white mice.* White outbred mice weighing 8–12 g were infected intranasally under light ether narcocis with 10-fold dilutions of the virus variants under study. The animals were observed for 12 days. The 50% lethal dose was calculated according to Reed and Muench.

*Dynamics of virus reproduction in the lungs of white mice.* Mice were infected intranasally with $10^5$ $EID_{50}$/0.05 ml of the virus. Lungs of 3 animals were sampled 3, 5 and 7 days post-infection (p.i.) to prepare 10% suspension in Eagle's medium. The supernatant was titrated in CE using a conventional method.

*Pathogenicity for CE.* Ten-fold dilutions of the viruses were inoculated into allantoic cavity of 10-day-old chick embryos. The embryos were examined daily for 7 days to register their death. $LD_{50}$ was calculated using the method of Reed and Muench, but embryos which died within 24 hr p.i. were not taken into account.

*Recombination test.* A recombination analysis used to detect ts mutations in individual genes of the variants under study was done in CE fibroblast cultures according to the method described (Ghendon *et al.*, 1981).

## *Results*

### *Reproduction of the cold-adapted variants of A/PR/8/34 virus in CE at 34° and 40 °C*

Table 1 shows the equal reproduction of the original A/PR/8/34 strain in CE at both the optimal (34 °C) and the non-permissive (40 °C) temperatures

**Table 1. Characterization of temperature-sensitivity and pathogenicity of cold-adapted variants of A/PR/8/34 influenza virus for white mice and CE**

| Virus | Reproduction in CE at temperatures | | Difference in titres at | Pathogenicity for | |
|---|---|---|---|---|---|
| | 34 °C | 40 °C | 34 °C and 40 °C | mice | CE |
| A/PR/8/34 | 9.75* | 9.75* | 0 | 4.5** | 8.8** |
| A/PR/8/30/25 | 9.5 | 7.25 | 2.25 | 4.2 | 8.8 |
| A/PR/8/40/25 | 8.75 | 4.0 | 4.75 | 2.0 | n. t. |
| A/PR/8/50/25 | 9.25 | 4.25 | 5.0 | 2.0 | n. t. |
| A/PR/8/60/25 | 9.5 | 4.0 | 5.5 | 1.6 | 4.8 |
| A/PR/8/59/1 | 9.75 | 1.75 | 8.0 | <1.0 | 4.2 |

* $\log_{10}$ $EID_{50}$/0.2 ml
** $\log_{10}$ $LD_{50}$/0.05 ml
n. t. — not tested

($RCT^{+}_{40}$). After 30 passages at 25 °C, a lower reproduction of the virus was noticed at 40 °C, the difference in titres at 34 ° and 40 °C being 2.25 log $EID_{50}$/0.2 ml. After 10 subsequent passages of A/PR/8/34 influenza virus at a low temperature a variant was selected, the titres of which at 40 °C were by 4.75 log lower than those at 34 °C. The variants A/PR/8/50/25 and A/PR/8/60/25 which underwent 50 or 60 passages at 25 °C, showed in their titres at 34° and 40 °C differences of 5.0 and 5.5 log, respectively.

A cold-adapted strain with a more marked ts phenotype was obtained as a result of a single cloning of A/PR/8/59/25 virus which had had 59 passages at 25 °C in CE using the limiting dilutions technique. The difference in the titres of this A/PR/8/59/1 clone at 34° and 40 °C took 8.0 log.

The variants studied retained a high reproductive capacity in CE at 34 °C throughout the process of adaptation to a low temperature and grew to the titres of 9.5—10.0 log $EID_{50}$/0.2 ml when inoculated into CE at a dose of $10^4$ $EID_{50}$/0.2 ml. Haemagglutinin content in the allantoic fluid was determined in CE inoculated with $10^1$—$10^6$ $EID_{50}$/0.2 ml of the virus. It was found that the original A/PR/8/34 strain and its cold-adapted variants including the A/PR/8/59/1 clone, were characterized by an equally high ability to produce haemagglutinin to the titres of 1 : 1024—1 : 8192. Thus, passages of A/PR/8/34 strain in CE at a low temperature resulted in the appearance of cold-adapted variants which reproduced poorly at 40 °C, but gave high yields in CE at 34 °C.

*Pathogenicity of the cold-adapted variants of A/PR/8/34 influenza virus for white mice and CE*

Table 1 summarizes the results of studies on pathogenicity of the original A/PR/8/34 strain and its cold-adapted variants for white mice. The original A/PR/8/34 strain was highly pathogenic for white mice (4.5 log $LD_{50}$/0.05 ml). The virus obtained after 30 passages at a low temperature was almost equally pathogenic. Subsequent passages of the virus in CE at 25 °C resulted in a decrease in its pathogenicity for white mice. Pathogenicity of the cold-

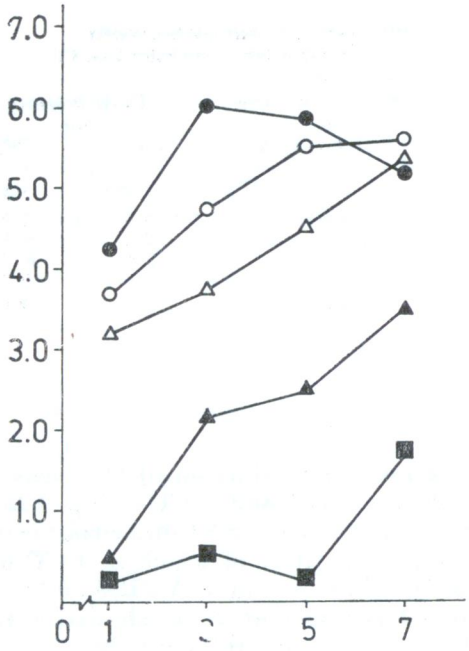


**Fig. 1.**
The dynamics of reproduction of the cold-adapted A/PR/8/34 virus variants in the lungs of white mice
The strains were obtained after initial (●—●), 30 (○—○), 40 (△—△) and 60 (▲—▲) passages of influenza A/PR/8/34 virus in CE at 25 °C. A/PR/8/59/1 cloned variant after 59 passages at low temperature (■—■).
Abscissa: period of observation (days); ordinate: virus titres (log $EID_{50}$/0.2 ml).

-adapted variants which underwent 40 and 50 passages at 25 °C was sufficiently low (2.0 log $LD_{50}$/0.05 ml). Further passages of A/PR/8/34 virus at 25 °C failed to reduce pathogenicity significantly, which was 1.6 log $LD_{50}$/0.05 ml after 60 passages. However, the cloned cold-adapted A/PR/8/59/1 variant has virtually lost its pathogenicity for mice ($< 1.0$ log $LD_{50}$/0.05 ml).

Studies on the dynamics of reproduction of the cold-adapted variants of A/PR/8/34 virus in the lungs of white mice revealed significant differences in the levels and patterns of accumulation of the viruses which had had a different number of passages in CE at a low temperature (Fig. 1). The original strain (0) was characterized by a high level of reproduction in the lungs of mice, the maximum concentration of the virus (6.0 log $EID_{50}$/0.2 ml) having been observed 3 days p.i. The variant with 30 passages at 25 °C reproduced in the lungs to the titres which were by 0.5 log lower than those of the original variant, the peak of its reproduction being observed on day 5. The cold-adapted variants of A/PR/8/34 obtained after 40 and 60 passages at 25 °C grew in the lungs to the titres of 5.5 and 3.5 log $EID_{50}$/0.2 ml, respectively, only by day 7. The lowest levels of reproduction in the lungs of mice were observed for the A/PR/8/59/1 clone that grew to maximum titres of 1.75 log $EID_{50}$/0.2 ml by day 7.

Studies on pathogenicity of the original A/PR/8/34 virus and its cold-adapted variants for CE have shown that pathogenicity of the original strain was 8.8 log $EID_{50}$/0.2 ml and persisted after 30 passages of the virus at 25 °C. A marked decrease in pathogenicity of A/PR/8/34 influenza virus

**Table 2. Recombination of the cold-adapted influenza A/PR/8/59/1 variant with FPV ts mutants**

| FPV mutants | Mutant genes | Mutant proteins | CE fibroblaststs double infected with FPV ts mutants and either A/PR/8/59/1 influenza virus or A/Krasnodar/101/59 virus at indicated temperatures | | | | CE fibroblasts infected with FPV ts mutants only at indicated temperatures (control) | |
|---|---|---|---|---|---|---|---|---|
| | | | A/PR/8/59/1 | | A/Krasnodar/101/59 | | | |
| | | | 36 °C | 40° or 42 °C | 36 °C | 40° or 42 °C | 36 °C | 40° or 42 °C |
| ts 29 | 1 | P3 | 7.7* | < 2.0* | 7.4* | 6.2* | 7.3* | < 2.0 |
| ts 131 | 2 | P1 | 7.8 | 6.0 | 7.7 | 6.4 | 7.7 | < 2.0 |
| ts 166 | 3 | P2 | 6.3 | < 2.0 | 6.1 | 5.2 | 6.4 | < 2.0 |
| ts US 1 | 5 | NP | 6.2 | < 2.0 | 7.7 | 5.6 | 7.9 | < 2.0 |
| ts 5 | 6 | NA | 6.7 | < 2.0 | 6.6 | 6.4 | 6.6 | < 2.0 |
| ts 303/1 | 7 | M | 6.3 | < 2.0 | 6.4 | 5.7 | 6.4 | < 2.0 |
| ts mN | 8 | NS | 7.1 | 6.2 | 7.0 | 6.3 | 7.3 | < 2.0 |

* $\log_{10}$ PFU/ml

for CE was detected after 60 passages at 25 °C. A minimum pathogenicity (4.2 log $EID_{50}$/0.2 ml) was observed with the cloned A/PR/8/59/1 variant.

*Analysis of ts mutations in the genome of the cloned cold-adapted variant of A/PR/8/34 virus*

Table 2 shows that the cold-adapted cloned A/PR/8/59/1 variant did not recombine with 5 of 7 FPV ts mutants, namely, with ts 29, ts 166, ts US 1, ts 5 and ts 303/1 having ts mutations in genes 1, 3, 5, 6 and 7 coding for P3, P2, NP, NA and M proteins, respectively. In control experiments with A/Krasnodar/101/59 virus, the genome of which contains no ts mutations (Ghenkina and Ghendon, 1979), all ts mutants showed a high level of recombination. At the same time, when CE fibroblast cultures were infected with FPV ts mutants only (without influenza virus), no plaques were formed at elevated temperature. These results suggest that genes 1, 3, 5, 6 and 7 coding for P3, P2, NP, NA and M proteins of the cold-adapted A/PR/8/59/1 variant, contain ts mutations.

## *Discussion*

The main aim of our investigations was to study the dynamics of alterations in biological properties (reproductive capacity at an elevated temperature, pathogenicity for mice and CE) of A/PR/8/34 influenza virus in a process of multiple passages in CE incubated at 25 °C. It was found that passages of this virus at low temperature led to a significant decrease in its ability to reproduce at elevated temperature (40 °C), and this correlated with a decrease in the level of pathogenicity for mice and embryos. However, these alterations in biological properties of the virus had no effect on its quality essential for the development of vaccine strains — a high reproductive capacity in CE at 34 °C.

The data on a decrease in pathogenicity of the cold-adapted variants of A/PR/8/34 influenza virus for mice are in agreement with similar results obtained by Maassab *et al.* (1972) who observed a significant decrease in pathogenicity of the above strain for mice after 60 passages at 33°, 31°, 28 °C, including 18 passages at 25 °C which had been carried out in tissue culture or CE. However, unlike to our variant, the cold-adapted variant selected by Maassab *et al.* (1972) reproduced in the lungs of mice nearly as efficiently as the original A/PR/8/34 virus.

The most marked ts phenotype and the lowest pathogenicity for mice and CE was observed with the cloned cold-adapted A/PR/8/59/1 variant which underwent a total of 60 passages in CE at 25 °C. The recombination test with FPV ts mutants belonging to 7 recombination-complementation groups, which had ts mutations in 6 genes coding for non-glycosylated proteins as well as in gene 6 coding for the neuraminidase, has shown that A/PR/8/59/1 variant contains ts mutations in 5 genes — 1, 3, 5, 6 and 7 responsible for the synthesis of P3, P2, NP, NA and M proteins, respectively.

Thus, multiple passages of A/PR/8/34 virus in CE at 25 °C followed by cloning resulted in obtaining the cold-adapted A/PR/8/59/1 variant that

acquired ts mutations in gene 6 responsible for the synthesis of the neuraminidase surface protein as well as in 4 other genes coding for the non-glycosylated P3, P2, NP, and M proteins. This variant drastically reduced its ability to reproduce in CE at 40 °C, decreased its pathogenicity for CE, lost its pathogenicity for mice by intranasal administration, greatly reduced its ability to reproduce in the lungs of mice, but retained a high reproductive capacity in CE at 34 °C.

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