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PARTIAL CHANGE OF THE TS-PHENOTYPE OF COLD-ADAPTED INFLUENZA VIRUS STRAIN GROWN IN CANINE KIDNEY CELLS IN THE PRESENCE OF TRYPSIN

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Summary. — The cold-adated temperature-sensitive (ts) influenza virus strain A/Leningrad/134/17/57 (H2N2) multiplied well at 32 °C (optimal temperature); lower titres of infectious virus were obtained in developing chick embryos at 40 °C. In a canine kidney (MDCK) cell line and in primary calf kidney (CK) cells an increased reproduction of the virus was found at 40 °C especially in the presence of trypsin. The ratios of virus titres obtained at optimal versus higher temperatures (RCT_{40}) were by 1,000 times lower than those found in chick embryos. Polyacrylamide gel electrophoresis revealed a comparable synthesis of the cold-adapted influenza virus strain polypeptides HA, NP, M and NS in MDCK cells, regardless whether they were incubated at optimal or non-permissive temperatures.

Key words: *influenza virus*; *ts-mutant*; *reproduction in cell culture*

Introduction

Adaptation of influenza virus to any susceptible host-cell system at lowered temperature of 25—28 °C leads to attenuation of the virus, which gains the ts-phenotype, i. e. it reveals a reduced reproduction at temperatures about 40 °C (Alexandrova and Smorodintsev, 1965; Maassab *et al.*, 1969).

In our studies, the ts-phenotype was determined in developing chick embryos which represent an optimal susceptible host-cell system for all epidemiologically important human influenza virus strains of A and B serotypes. Differences in virus titres obtained at 32 and 40 °C (RCT_{40}) were as high as 6.0—7.5 log units EID_{50}/0.2 ml (Alexandrova *et al.*, 1979). Analogous foreign studies were often performed in chick kidney primary cultures or cell lines (Maassab *et al.*, 1969), in monkey kidney or in calf kidney (CK) cells (Mills and Chanock, 1971; Murphy *et al.*, 1978). Recently an established line of canine kidney (MDCK) cells, in which at optimal temperature titres of the laboratory strain of influenza virus were similar to values obtained in developing chick embryos, became widely used (Tobita *et al.*, 1975;

Markoff *et al.*, 1979; Oxford *et al.*, 1980). The aim of the present study was to compare the reproduction of cold-adapted influenza virus strain A/Leningrad/134/17/57 in chick embryos, in MDCK cells and in primary CK cells incubated at 32 and 40 °C, respectively.

Materials and Methods

Viruses. The cold-adapted influenza virus strain A/Leningrad/134/17/57 (H2N2) had undergone 25 chick embryo passages at optimal temperature (32 °C) followed by 17 passages at 25—28 °C. The epidemic influenza virus strain A/Leningrad/538/74 (H3N2) of type A/Port Chalmers had undergone 4 chick embryo passages at 32 °C.

Cell cultures. MDCK and CK cells were obtained from the Cell Culture Laboratory, Influenza Research Institute, the U.S.S.R. Ministry of Health, Moscow, U.S.S.R.

Assay of virus infectivity in cell cultures. Cells were infected with tenfold virus dilutions and incubated for 48 hr at 32 and 40 °C, respectively. The presence of infectious virus was determined by inoculation of chick embryos with undiluted culture fluids.

Trypsin used in a final concentration of 3 μg/ml was received from the Tomsk Institute of Vaccines and Sera, the U.S.S.R. Ministry of Health.

Determination of the ts-phenotype was performed by determination of the RCT_{40} marker, i. e. the difference between virus titres in cell cultures and eveloping chick embryos incubated for 48 hr at 32 and 40 °C.

Synthesis of virus-specific proteins was followed in MDCK cells infected at multiplicity of 0.5—1.0 EID_{50}/cell. The virus was allowed to adsorb for one hour at 36 °C in a minimal volume of Eagle's medium. Infected cells were incubated for 4 hr at 32 or 40 °C in complete Eagle's medium, then refed for 1 hr with an incomplete medium lacking the amino acids leucine, glycine and alanine. Cells were labelled for one hr in the presence of these ^3H-labelled amino acids, (370 kBq/ml) either at 32 or at 40 °C. Unincorporated labelled amino acids were thoroughly washed off by cold phosphate buffered saline. The cells were then collected by scraping, centrifuged and the cell pellet was lysed by 2% sodium dodecyl sulphate solution containing 5 mol/l urea and 50 mol/l dithiothreitol. The cell lysate was electrophoresed in 7.5% polyacrylamide gel according to Laemmli (1970). The autoradiograms in 1—2 mm gel segments were evaluated in a "Nuclear Chicago Mark-1" counter.

Results

Comparison of influenza virus reproduction in chick embryos and cell cultures at 32 and 40 °C

The low-passaged wild-type influenza virus strain A/Leningrad/538/74 multiplied similarly well in developing chick embryos at both optimal (32 °C) and higher (40 °C) temperatures (Table 1). The difference in virus titres was not higher than 0.5 log unit. The cold-adapted strain A/Leningrad/ /134/17/57 multiplied well at 32 °C, but its titre at 40 °C was markedly reduced (by 7.0 log units).

In MDCK cells, the wild-type strain A/Leningrad/538/74 multiplied similarly well at either cultivation temperature: the difference in virus titres at the optimal (32 °C) and non-permissive (40 °C) temperatures did not exceed 0.7 log unit, being similar to the RCT_{40} marker obtained in developing chick embryos. Though the yield of ts strain A/Leningrad/134/17/57 at optimal temperature was by 1.9 log units lower, at 40 °C it was by 1.7 log units higher in MDCK cells than in chick embryos. The difference in virus titres in MDCK cells cultivated at 32 and 40 °C reached 3.4 log units, i. e.

Table 1. Temperature-sensitivity of influenza virus strains assayed in developing chick embryos and cell cultures

Host cell system	Virus strain	Virus titres					
		Trypsin absent			Trypsin present		
		32 °C	40 °C	RCT_{40}**	32 °C	40 °C	RCT_{40}
Developing chick embryos	A/Leningrad/538/74	6.5*	6.0	0.5	not tested		
	A/Leningrad/134/17/57	7.8	0.8	7.0	not tested		
MDCK cell line	A/Leningrad/538/74	6.7	6.0	0.7	7.2	6.3	0.9
	A/Leningrad/134/17/57	5.9	2.5	3.4	5.5	2.9	2.6
CK primary cell cultures	A/Leningrad/134/17/57	6.4	2.4	4.0	6.3	3.0	3.3

* log EID_{50}/0.2 ml (mean values from 3-4 experiments).
** Titre ratio: at 32 °C/at 40 °C.

it was by 3.6 log units lower than the value of the RCT_{40} marker found in chick embryos.

Analogous reduction of the temperature sensitivity index of cold-adapted strain A/Leningrad/134/17/57 was observed in primary CK cell cultures in which the RCT_{40} marker reached the value of 4.0 log units as compared with that of 7.0 log units obtained in chick embryos.

Effect of trypsin on reproduction of the cold-adapted influenza virus strain in cell cultures

It is known that trypsin increases the formation of infectious virions of influenza virus in some cell cultures due to the cleavage of the haemagglutinin precursor to HA_1 and HA_2 polypeptides (Lazarowitz *et al.*, 1973; Klenk *et al.*, 1975). Because reproduction of the cold-adapted virus strain A/Leningrad/134/17/57 at optimal temperature was about 1,000 times lower in MDCK and CK cells than in developing chick embryos, we studied whether trypsin would affect its reproduction in these cultures.

As shown in Table 1, addition of trypsin to the maintenance medium did significantly influence the reproduction neither of cold-adapted strain nor of wild-type strain A/Leningrad/538/74 at optimal temperature. Reproduction od the latter strain was neither substantially affected at 40 °C, so that the RCT_{40} marker preserved its minimal importance. Contrasting results were obtained with the cold-adapted strain A/Leningrad/134/17/57, the reproduction of which was reduced in the presence of trypsin at 32 °C by 0.4 log unit (to 5.5 EID_{50}/0.2 ml) in MDCK cell cultures and by 0.1 log unit (to 6.3 EID_{50}/0.2 ml in CK cell cultures. At the same time, virus titres in the presence of trypsin at non-permissive temperature increased in MDCK as well as in CK cell cultures by 0.4 and 0.6 log units, respectively. In connection with these results, differences in virus titres at 32 °C and 40 °C in CK and MDCK cell cultures were reduced from 4.0 to 3.3 and from 3.4 to 2.6 log units, respectively.

The results obtained point out the influence of cultivation conditions on the RCT_{40} marker of cold-adapted virus strains. The most pronounced differences in virus reproduction at optimal and higher temperatures can

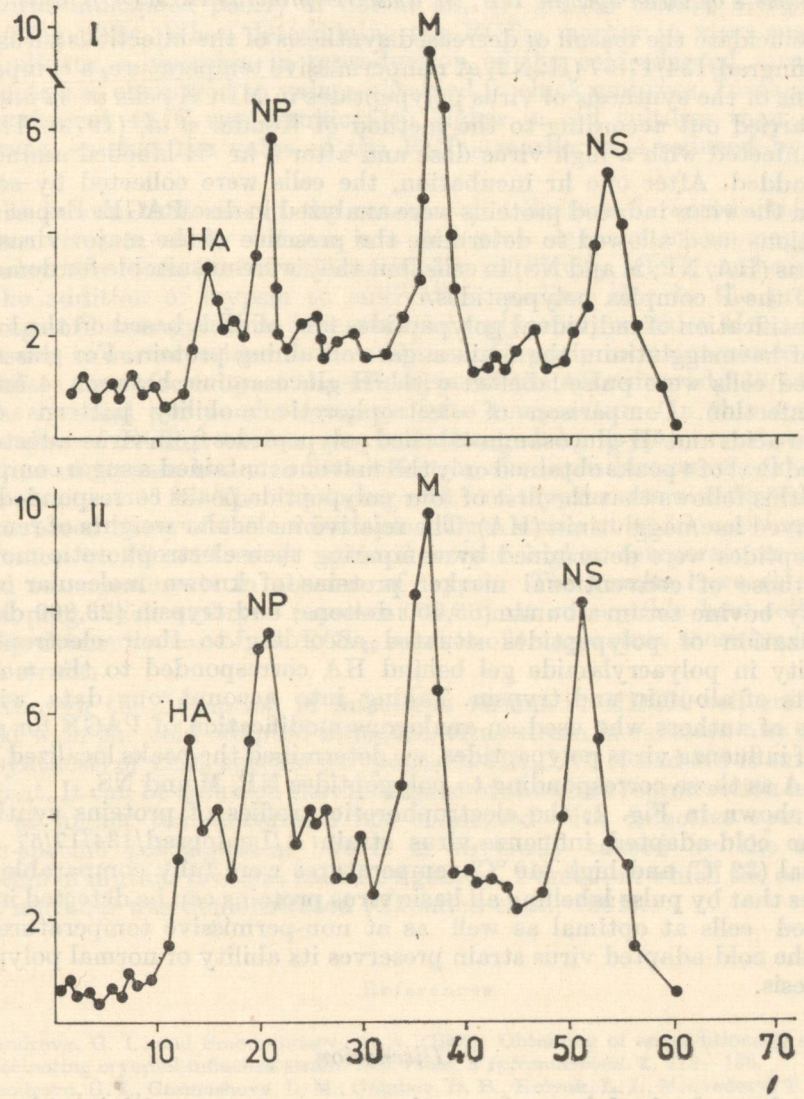

Fig. 1.

Electrophoretic profile of virus-specific proteins synthesized in MDCK cells infected with influenza virus strain A/Leningrad/134/17/57 (H2N2) incubated at 32 °C (I) and 40 °C (II) Abscissae: fraction numbers; ordinates: incorporation of ^3H-labelled amino acids (counts/min × × 10^{-3}).

be found in developing chick embryos, whereas in MDCK and CK cell cultures a marked reduction of the RCT_{40} marker occurs even in the presence of trypsin.

Synthesis of virus-specific NP, M and NS proteins in MDCK cells

To elucidate the reason of decreased synthesis of the infectious virus strain A/Leningrad/134/17/57 (H2N2) at nonpermissive temperature, a comparative analysis of the synthesis of virus polypeptides in MDCK cells at 32 and 40 °C was carried out according to the method of Kendal *et al.* (1973). The cells were infected with a high virus dose and after 5 hr ^{3}H-labelled amino acids were added. After one hr incubation, the cells were collected by scraping off and the virus induced proteins were analyzed in disc PAGE. Experimental conditions used allowed to determine the presence of the major virus-coded proteins (HA, NP, M and NS) in cells, but they were unsuitable for demonstration of the P complex polypeptides.

Identification of individual polypeptides first of all is based on the localization of haemagglutinin, the main sugar-containing protein. For this reason, infected cells were pulse-labelled with ^{3}H-glucosamine between 4 and 5 hr post-infection. Comparison of electrophoretic mobility patterns of ^{3}H--amino acids and ^{3}H-glucosamin-labelled polypeptides from virus-infected cells showed that of 4 peaks obtained only the first one contained a sugar component. From this follows that the first of four polypeptide peaks corresponded to the uncleaved haemagglutinin (HA). The relative molecular weights of remaining polypeptides were determined by comparing their electrophoretic mobilities with those of conventional marker proteins of known molecular weight, namely bovine serum albumin (65,000 daltons) and trypsin (23,800 daltons). Localization of polypeptides situated according to their electrophoretic mobility in polyacrylamide gel behind HA corresponded to the molecular weights of albumin and trypsin. Taking into account our data with the results of authors who used an analogous modification of PAGE for separation of influenza virus polypeptides, we determined the peaks localized behind the HA as those corresponding to polypeptides NP, M and NS.

As shown in Fig. 1, the electrophoretic profiles of proteins synthesized by the cold-adapted influenza virus strain A/Leningrad/134/17/57 at the optimal (32 °C) and high (40 °C) temperatures were fully comparable. It indicates that by pulse labelling all basic virus proteins can be detected in virus-infected cells at optimal as well as at non-permissive temperatures, i. e. that the cold-adapted virus strain preserves its ability of normal polypeptide synthesis.

Discussion

The data obtained have shown that temperature-sensitivity (the RCT_{40} marker) of influenza virus strain A/Leningrad/134/17/57 (H2N2), adapted by prolonged passaging at lower temperatures to cultivation conditions at 25—28 °C, varied depending on the host cell system used for determination

of the RCT_{40} marker. The maximum difference of virus reproduction at optimal versus higher temperatures was found in experiments on developing chick embryos — the most susceptible host cell system, in which the titre of the cold-adapted virus strain reached 7.8 log units EID_{50}/0.2 ml. At 40 °C the virus multiplied poorly or not at all, the RCT_{40} marker being as high as 7.0 log units. When determining the RCT_{40} marker in virus-susceptible cell cultures, a decrease in reproduction of the cold-adapted virus strain was noted as compared to values obtained in chick embryos. In parallel, the virus titre at 40 °C was significantly higher in cell cultures than in chick embryos, so that the value of the RCT_{40} marker was reduced by 3.6 log units.

It is known that one of the reasons of a decreased reproduction of influenza virus in some cultures is a defective cleavage of haemagglutinin. It leads to the formation of virions with low infectivity, which can be increased by the addition of trypsin to cultivation medium due to the proteolytic cleavage of haemagglutinin precursor into glycopolypeptides HA_1 and HA_2. However, in our experiments the addition of trypsin to maintenance medium did affect the infectivity of the wild-type strain A/Leningrad/538/74 neither at the optimal nor at the nonpermissive temperature. In this connection the value of RCT_{40} marker was not changed by trypsin treatment. The addition of trypsin had also little effect on the reproduction of cold-adapted virus strain at 32 °C. The enhancing effect of trypsin was manifested mainly by increased titres of the cold-adapted virus strain at 40 °C, causing a lower difference in virus titres at optimal versus higher temperatures as compared to experiments in absence of trypsin. The results have shown that developing chick embryos represent the most susceptible host-cell system for the determination of the RCT_{40} marker of temperature sensitive influenza virus strains.

Although the formation of infectious virions in MDCK cell cultures infected with the cold-adapted influenza virus strain is substantially reduced, the synthesis of virus-specific polypeptides HA, NP, M nad NS is sufficiently efficient. It can be assumed that reduction of the virus reproduction in MDCK cells at higher temperature is not connected with defective synthesis of virus-specific polypeptides, but it is obviously caused by the impaired function of mutant proteins coded by genes 1, 5 and 7, in which the occurrence of ts mutants was demonstrated (Ghendon *et al.*, 1981).

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