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INTERACTION OF ŤAHYŇA VIRUS WITH DIPLOID HUMAN EMBRYO FIBROBLASTS IN VITRO

I. SCHWANZEROVÁ

Research Institute of Epidemiology and Microbiology, Bratislava, Czechoslovakia

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Summary. — Reproduction in human diploid cell strains (HDCS) of 2 variants of Ťahyňa virus strain 236 was compared. In the first passage, the extraneural (E) variant of strain 236 multiplied intensively in this cell system, while the neuro-adapted (N) variant of strain 236 multiplied less intensively. In spite of their starting infectivity, neither virus variant could be passed by transfer of free infective culture fluid. A cytopathic effect (CPE) resulted after inoculation of intact cell monolayers with infected cells. No significant differences were found in the response of several HDCS to infection with the two virus variants.

Introduction

In previous studies on Ťahyňa virus in vitro, Šefčovičová (1962*a*, 1964, 1965) investigated its relations to primary cultures of some avian and vertebrate cells and to heteroploid cell lines derived from normal or malignant tissues of primates and lower vertebrates. These investigations were mainly aimed at elucidating the biological properties of Ťahyňa virus and at establishing a suitable cell system for primary isolation of the virus.

Based on the properties of HDCS (Hayflick and Moorhead, 1961), we assumed that investigations of Ťahyňa virus growth in these normal human fibroblasts in vitro could contribute to the elucidation of its interaction with cells in the intact organism and thus of some aspects of its pathogenesis.

The E variant of Ťahyňa virus strain 236, isolated by intramuscular inoculation of Syrian hamsters (Bárdoš, 1961) and further passed by means of their viraemic blood, probably, resembles the virus circulating in nature and, under laboratory conditions, considerably differs by certain biological properties from virus strains passed in a different way (Šefčovičová, 1962*b*; Wallnerová and Albrecht, 1966).

After having estabilished the possibility of propagating Ťahyňa virus in HDCS (Schwanzerová, 1967), further work was mainly directed to the E variant. The present report deals with comparative investigations on the growth properties of both E and N variants of strain 236 in human embryo fibroblasts.

Materials and Methods

The HDCS strains used are listed in Table 1. Strain WI-38 (Hayflick and Moorhead, 1961) was obtained from Dr. Trlifajová, Institute of Epidemiology and Microbiology, Prague. Strain LEP_{13} was derived from the lungs of a 2—3 months old human embryo at the Institute of Sera and Vaccines, Prague (Řezáčová, personal communication); cells with regularly controlled

chromosome numbers were shipped from Prague to our laboratory once weekly in suspension, which was seeded within 24 hours after shipment. The RIEM (Research Institute of Epidemiology and Microbiology, Bratislava) strains were derived from various tissues of 8—12 weeks old human embryos obtained in interruptions of gravidity in apparently healthy women; all were of the fibroblast type. The RIEM-16 strain was mainly epithelial-like in the first passages, after which islets of fibroblasts rapidly multiplied and finally formed a continuous monolayer. All RIEM strains were subcultured at 4—5 days' intervals in 500- and 1200-ml Roux bottles. The monolayers, after removal of medium, were overlaid with trypsin solution heated to 37° C. After 1—2 minutes, the trypsin solution was removed and the cultures were kept at room temperature or at 37° C until all cells became detached from the glass. Subpassaging was carried out in the ratio of 1 : 2. Tube cultures for virus titration and virus growth curve experiments were seeded with 10^5 cells in 1 ml of medium and incubated for 3 days at 37° C in a stationary position.

The GMK-AH-1 cell line, derived from *Cercopithecus aethiops* monkey kidneys, has been maintained in the Virology Dept. of this Institute for several years. Tube cultures for virus titration were seeded with $(4—5) \times 10^4$ cells in 1 ml medium and incubated for 48—72 hours at 37° C.

Media. Stock HDCS cultures were grown in medium consisting of 2 parts Eagle's basal medium with 10% calf serum and 1 part of 0.5% lactalbumin hydrolysate in Hanks' solution. Medium EPL, supplied from the Institute of Sera and Vaccines, Prague (Michl and Řezáčová, 1966), was used as growth medium for all tube and bottle HDCS cultures destined for virus inoculation. Tube cultures of GMK cells were grown in synthetic medium ÚSOL (Slonim *et al.*, 1960; supplied by the Institute of Sera and Vaccines, Prague) supplemented with 10% calf serum. As maintenance media were used: medium EPL for HDCS and synthetic medium ÚSOL with 2% calf serum for GMK cells. The pH of all media was adjusted to 7.5—7.6 with a 7.5% solution of $NaHCO_3$. The growth medium for HDCS stock cultures contained 50 μg/ml aureomycin, the other media contained 100 units penicillin and 100 μg streptomycin per ml.

Trypsin (Difco 1 : 250) was used as a 0.25% solution in phosphate buffered saline.

Virus. The neuroadapted (N) and extraneural (E) variants of Ťahyňa virus strain 236 used in the experiments had undergone 17—19 brain passages in young or suckling mice and 8—10 intramuscular passages in young Syrian hamsters, respectively.

Inoculation of HDCS. 1) *Titration in tubes.* Each of 4 confluent tube cultures was inoculated with 0.1 ml of the given virus dilution. The cultures were incubated in a stationary position at 37° C. The CPE was read 5—7 days after inoculation and the titres were expressed in CPD_{50} values. 2) *The virus growth curves* were determined in a series of tube cultures inoculated with 0.1 ml of virus suspensions at different input multiplicities of infection. After 30 minutes' adsorption at 37° C, the medium was removed and the cultures were washed thrice with Hanks' solution, supplied with 1 ml fresh medium and incubated further at 37° C. At intervals, the levels of virus in medium were determined in tube cultures of GMK cells of in intracerebrally inoculated 6—7 g white mice. 3) *Passaging of virus.* Fresh monolayer cell cultures in Blake bottles were inoculated with 1.2 ml of infectious medium (undiluted or diluted 1 : 10) from the preceding passage or with a part of infected cells (1/10—1/2 of the total amount of cells) suspended in 1.2 ml of medium and 10.8 ml medium was then added. The virus titres in medium were determined in tube cultures of GMK cells.

Results

Cytopathic activities of the N and E variants of strain 236 in different HDCS

The susceptibility of different HDCS to the two virus variants was estimated by comparing the CPD_{50} titres attained in the individual cell strains and the LD_{50} titres determined intracerebrally in young mice.

Table 2 shows that no CPE was observed in cell cultures inoculated with the E virus variant. These cultures showed no morphological changes within 12 days of cultivation, the medium being changed every 3—4 days. The N variant of virus produced a CPE only at a high input multiplicity of infection. The CPE was frequently irregular and incomplete.

Table 1. Characteristics of the human diploid cell strains employed

Strain	Embryo No.	Tissue	Used in passage No.
WI-38		Lung	35—40
LEP_{13}		Lung	12—25
RIEM-2	2	Skin + muscle	8
RIEM-3	3	Lung	9—20
RIEM-4	4	Whole embryo	12—20
RIEM-16	15	Kidney	6—10
RIEM-24	20	Skin + muscle	4—10
RIEM-25	20	Lung	4—10
RIEM-28	22	Skin + muscle	4—30
RIEM-29	22	Heart	6—10
RIEM-32	22	Liver	6—10

Multiplication of the N and E variants of strain 236 in HDCS

In spite of their low cytopathic activities, both virus variants multiplied well in human fibroblasts (Fig. 1). Release of virus into medium, assayed by intracerebral inoculation of young mice and in GMK cell cultures, started in both variants between the 6th and 9th hour after the end of adsorption, reaching a maximum between the 24th and 72nd hour, depending on the input multiplicity of infection.

Table 2. Comparative titration of two variants of Ťahyňa virus strain 236 in HDCS and mice

Virus variant	Exp. No.	Virus titres in						
		HDCS (log CPD_{50}/0.1 ml)						Mice (log ic LD_{50}/0.03 ml)
		WI-38	LEP_{13}	RIEM-2	RIEM-3	RIEM-4	RIEM-16	
236-E	1	< 0.7						7.2
	2		< 0.7	< 0.7				7.0
	3				< 0.7	< 0.7		3.5
	4		< 0.7		< 0.7	< 0.7		4.5
	5						< 0.7	4.0
236 N	1	2.0						6.5
	2		2.3	2.0				6.3
	3				2.5	2.5		> 7.3
	4		2.5		2.5	2.5		7.5
	5			2.7				7.2

The limiting dose of inoculum capable of inducing the multiplication of either virus variant corresponded approximately to input multiplicities of infection between 0.001—0.01 ic mouse LD_{50} per cell. The E virus variant reached in the medium 100—1000 times higher titres than the N variant at the same intervals (Table 3).

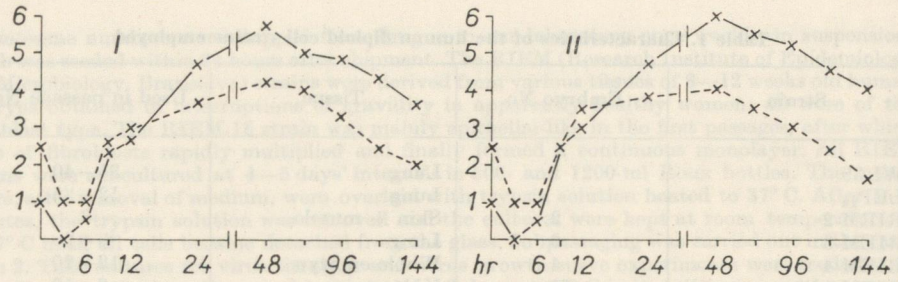

Fig. 1.

Multiplication of the extraneural (———) and neuroadapted (— — —) variants of Ťahyňa virus strain 236 in LEP_{13} cells
Input multiplicity of infection 10 LD_{50} per cell.
Abscissa: hours after the end of adsorption
Ordinate: virus titres in log LD_{50}/0.03 ml (I) and log GMK CPD_{50}/0.1 ml (II) values

Comparison of the susceptibility of different HDCS to the N and E virus variants

The susceptibilities to the two virus variants of different HDCS, derived either from the same organs of different embryos or from different organs of the same embryo, were compared in parallel or independent experiments, the results of which are summarized in Table 4. No significant differences were found between either different cell strains or cells used at different passage levels.

Table 3. Multiplication of two variants of Ťahyňa virus strain 236 in HDCS after different input multiplicities of infection (MI)

MI (GMK CPD_{50} per cell)	Virus titres (log GMK CPD_{50}/0.1 ml)					
	E variant			N variant		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
10	N. d.	N. d.	N. d.	4.0	3.5	1.5
1	3.5	5.0	> 6.5	3.3	3.5	3.0
0.1	2.5	5.0	5.5	3.0	2.7	2.0
0.01	2.5	3.5	5.0	2.0	2.7	2.3
0.001	< 0.7	< 0.7	< 0.7	< 0.7	< 0.7	< 0.7

N. d. = not done.

Passaging in HDCS of the N and E virus variants

Adaptation of the two virus variants to human embryo fibroblasts by serial passaging was attempted in two ways. The usual mode of passaging by transfer of a part of infectious fluid from cell cultures 48—72 hours after inoculation into fresh homologous cultures led with both variants to a gradual decrease in virus titres in the medium until complete disappearance of virus in the 5th or 6th passage. The infected cells showed no morphological changes in comparison with controls (Table 5).

In the second method of passaging, infected cells from the preceding passage were used. In this case, total lysis of cells occurred in the 3rd—4th passage, the titres of virus in the medium reaching values of 10^4—10^5 ic mouse LD_{50} with the E variant and 10^1 ic mouse LD_{50} with the N variant (Table 6).

Table 4. Multiplication of two variants of Ťahyňa virus strain 236 in various HDCS after different input multiplicities of infection (MI)

Cell strain (passage level)	MI (GMK CPD_{50} per cell)	Virus titre in medium 3 days after inoculation (log GMK CPD_{50}/0.1 ml)	
		E variant	N variant
LEP_{13}	10	5.3	3.0
(19th pass.)	1	N. d.	3.0
	0.1	6.0	2.7
	0.01	> 6.3	3.0
RIEM-4	1	N. d.	2.7
(14th pass.)	0.01	6.3	N. d.
RIEM-16	0.1	> 5.0	N. d.
(8th pass.)			
RIEM-24	10	N. d.	2.0
(4th pass.)	1	N. d.	2.5
	0.1	N. d.	2.3
	0.01	N. d.	2.5
RIEM-25	10	N. d.	> 2.5
(9th pass.)	1	5.3	> 2.5
	0.1	5.0	> 1.5
	0.01	5.5	> 1.5
RIEM-28	10	N. d.	> 2.5
(6th pass.)	1	6.5	2.3
	0.1	5.5	2.5
	0.01	5.0	2.5
RIEM-29	10	N. d.	2.3
(10th pass.)	1	5.5	2.3
	0.1	5.0	1.7
	0.01	4.5	1.7
RIEM-32	10	N. d.	> 2.5
(10th pass.)	1	5.5	2.5
	0.1	5.0	2.0
	0.01	5.3	2.3

N. d. = not done.

Attempt at inducing persistent infection of HDCS with the E virus variant

In attempts at inducing persistent infection of RIEM-28 cells, monolayer cultures in Blake bottles were inoculated with the E virus variant at input

Table 5. Passaging of two variants of Ťahyňa virus strain 236 in LEP_{13} cell by transfer of medium from infected cultures

Passage No.	E variant		N variant	
	Virus titre in medium*	CPE	Virus titre in medium*	CPE
1	5.7	—	3.0	±
2	5.0	—	1.5	—
3	3.5	—	1.5	—
4	2.5	—	< 0.7	—
5	2.0	—	< 0.7	—
6	< 0.7	—	Not done	
7	< 0.7	—	Not done	

* log ic mouse LD_{50}/0.03 ml.
— = CPE absent.

multiplicities of infection of 0.03, 0.003 and 0.0003 GMK CPD_{50} of virus per cell, respectively. The infected cells were then passed in the same way as uninfected controls. The levels of infectious virus in medium were determined at each passage. In repeated experiments we failed to induce a persistent infection. In all cases, total cell degeneration occurred as early as in the

Table 6. Passaging of two variants of Ťahyňa virus strain 236 in LEP_{13} cells by transfer of infected cells

Passage No.	E variant		N variant	
	Virus titre in medium*	CPE	Virus titre in medium*	CPE
1	4.0	—	3.7	—
2	3.7	++	1.5	—
3	4.3	++++	1.0	—
4	Not done		1.0	++++

++++ = total CPE. For other explanations see Table 5.

2nd passage. Infected cells kept in Blake bottles by regular change of medium every 4—5 days produced infectious virus for more than 20 days, after which the cultures gradually degenerated.

Discussion

The E variant of Ťahyňa virus strain 236 reproduced rather weakly in the primary passages in most of the cell systems studied, virus multiplication usually being not accompanied by a CPE.

However, the virus could be passed in these cells; cytopathic viral particles evidently became prevalent and a CPE appeared after a few passages (Šefčo-

vičová, 1964, 1965). In marked contrast to other cell systems, the GMK-AH-1 cell line strongly supports multiplication of the E variant of strain 236 with the formation of a regular and complete CPE (Šefčovičová and Wallnerová, 1967).

Infection of HDCS with the E variant of strain 236 did not lead to CPE formation. But, as compared with the N variant, the multiplication in the primary passage of the E virus was more intensive. Its titres in the culture fluid were 100—1000 times higher than those of the N variant. In this way human embryo fibroblasts differ from the other cell systems which showed a higher susceptibility in particular to the N variant (Šefčovičová, 1964). The results obtained in experiments on the multiplication in HDCS of the N variant of strain 236 are in accordance with previous ones on the multiplication in HDCS of the neuroadapted line of strain 92 of Ťahyňa virus (Schwanzerová, 1967).

In spite of the good adsorption ability of either variant of strain 236 onto both HDCS and human tissue in vitro (Schwanzer, unpublished results), and in spite of that several stages of viral infection, ending in release of infectious virus into the culture fluid had been completed, serial passaging of the virus by transfer of culture fluid failed in the diploid cell system. A similar phenomenon was reported for rabies virus in WI-38 cells, but in this case already in the primary passage fluorescent antibody technique revealed a very low percentage of infected cells releasing a low amount of infectious virus into the culture fluid (Wiktor *et al.*, 1964). Nevertheless, these authors succeeded in adapting rabies virus to HDCS by the cell-mixing transfer technique.

The question arises as to how to explain and correlate the individual experimental results, namely the drop in virus titre in the course of passaging by the culture fluid and the absence of the CPE in this mode of passaging in contrast to the possibility of inducing the CPE by transfer of infected cells. It could be assumed that the virus released from cells into the medium may qualitatively differ from intracellular virus, namely in that it represents an inhomogeneous population of particles, including incomplete viral particles with changed specific activities towards individual cell systems and the role of which rapidly becomes prevalent in the course of pasaging.

Another possible explanation of the absence of virus from the culture fluid could be a disturbance in the release of virus from cells. This assumption is indirectly supported by the results of previous experiments (Schwanzerová, 1967), in which comparatively high levels of intracellular virus were found in diploid cells infected with two lines of strain 92 of Ťahyňa virus.

Nor can an effect of antiviral substances in the infected cell be excluded, because the production of interferon or other virus inhibitors was not investigated.

With the aim to detect differences in cell response to viral infection, cell strains derived from different organs of the same embryo and from the same organs of different embryos were examined. The response to Ťahyňa virus infection was almost uniform in all cases so that the present results offered no evidence of a different susceptibility of HDCS of different origin.

We failed to induce persistent infection of HDCS (strain RIEM-28) with

Ťahyňa virus. It has been suggested, however, that individual HDCS differ from one another with respect to the possibility of inducing a persistent infection (Libíková and Motajová, 1967).

References

Bárdoš, V. (1961): The Ťahyňa virus. I. Study of its resistance to the action of some physical factors and chemical agents. *Acta virol.* **5**, 50—56.

Hayflick, L., and Moorhead, P. S. (1961): The serial cultivation of human diploid cell strains. *Exp. Cell Res.* **25**, 585—621.

Libíková, H., and Motajová, J. (1967): Persistent infection of a human diploid cell strain with tick-borne encephalitis virus. *Acta virol.* **11**, 380.

Michl, J., and Řezáčová, D. (1966): Cultivation of mammalian cells in a medium with growth-promoting proteins from calf serum. *Acta virol.* **10**, 254—259.

Schwanzerová, I. (1967): Reproduction of Ťahyňa virus in human diploid cells. *Acta virol.* **11**, 63—64.

Šefčovičová, S. (1962*a*): Some data on the cultivation o Ťahyňa virus in tissue cultures. *Acta virol.* **6** 32—36.

Šefčovičová, L. (1962*b*): A comparison of two Ťahyňa virus variants in tissue culture. *Acta cirol.* **6**, 280.

Šefčovičová, L. (1964): Cultivation of Ťahyňa virus in cell cultures (in Slovak). Thesis. Institute of Virology, Bratislava.

Šefčovičová, L. (1965): Cultivation of Ťahyňa virus in stable cell lines. *Acta virol.* **9**, 495—501.

Šefčovičová, L., and Wallnerová, Z. (1967): Cytopathic activity of the extraneural variant of Ťahyňa virus in the GMK cell line. *Acta virol.* **11**, 270.

Slonim, D., Michl, J., Cinnerová, O., Mareš, J., and Dřevo, M. (1960): Some experiences with the preparation of inactivated vaccine against poliomyelitis in Czechoslovakia. II. Preparation of media (in Czech). *Čs. Epidem.* **9**, 111—121.

Wallnerová, Z., and Albrecht, P. (1966): Reproduction of Ťahyňa virus in chick embryo cell cultures studied by biological and morphological methods. *Acta virol.* **10**, 140—148.

Wiktor, T. J., Fernandes, M. V., and Koprowski, H. (1964): Cultivation of rabies virus in human diploid cell strain WI-38. *J. Immunol.* **93**, 353—366.