

FURTHER STUDIES ON INTERFERON AND VIRUS PRODUCTION IN YOUNG AND OLD CELL CULTURES PREPARED FROM ORDINARY AND RIF-FREE CHICK EMBRYOS

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Summary. — Experiments on chick embryo cells (CEC) prepared from RIF-free chick embryos confirmed the previous observation that in CEC cultures grown *in vitro* for seven days, interferon (IF) production was higher and tick-borne encephalitis (TE) virus titre lower than in cultures 24 hours old at the moment of infection. The results were consistent with those obtained in ordinary (probably RIF-positive) CEC and ruled out the possibility that RIF could be responsible for the effect of age. Nor was contact inhibition an immediate cause of the effect in old cultures: suspension CEC cultures prepared from 7 days old monolayers yielded more IF and less TE virus than cultures prepared from 1-day old monolayers (cells from both RIF-free and ordinary embryos). Cell density in 1-day old monolayers affected IF yield per cell only very little; a slightly higher IF production was detected in 10-fold denser cultures. Viral antigen was detected by immunofluorescence virtually in all cells in young cultures but at most in 50% of cells in old cultures.

Introduction

A positive correlation between the age of chick embryos, cell cultures or animals, serving as a source of the cells, and the production of IF was reported by several authors (for references see Henslová and Libíková, 1966; Libíková and Henslová, 1969). This phenomenon, as well as the negative correlation between the age and virus production was investigated in our laboratory on the system CEC—TE virus. In our previous experiments (Libíková and Henslová, 1969), we employed chick embryos from a flock which was not controlled for the presence of avian tumour viruses. Therefore the question arose as to whether actually RIF could have exerted some effect on IF production in aging cultures. CEC suspensions were pools prepared from several embryos. If only a small proportion of embryos contained the RIF, this could have infected all the cells within 7 days. Therefore we felt it necessary to repeat the experiments by using RIF-free embryos for the preparation of CEC, although to our knowledge there is no evidence that RIF would interfere with any virus other than a corresponding Rous sarcoma virus (Bryan) pseudotype.

We were also interested in two more questions: 1) does the effect of *in vitro* age persist even in suspension culture of cells recovered from a 7 days old monolayer?; and 2) does cell density influence IF production in 1-day old cultures?

In the present work we used immunofluorescence as a further criterion of virus reproduction, although the relatively low sensitivity of this technique presents definite limitations for interpretation of the results.

Materials and Methods

Chick embryos. RIF-free eggs were obtained from the Institute of Experimental Biology and Genetics, Prague (Dr. I. Hložánek), and in a smaller amount from the Institut Gustave Roussy, Villejuif, France (courtesy of Dr. J. Huppert). Ordinary eggs were purchased from the farm in Miloslavov (Slovakia).

Cell cultures. CEC suspensions were prepared from 10 days old embryos; in most cases they were pools from several embryos. The "Synthetic TC medium" (purchased from the Institute of Sera and Vaccines, Prague), supplemented with 5 or 10% of heated calf serum was used as both growth and maintenance medium. Unless otherwise stated, cultures were seeded with a suspension of $0.8-1.0 \times 10^6$ cells/ml. Stationary cultures were grown in tubes or Roux bottles. Suspension cultures were stirred by magnets.

Viruses. TE virus of the Western subtype, Hypr strain, Hy-M line, at the 50th-52nd mouse cerebral passage level, was used throughout. Western equine encephalomyelitis (WEE) virus, strain 15, was obtained from the collection of this Institute. Infectious tissue culture fluids from CEC cultures served as virus stocks.

Infection of the cultures and materials for assay. Before infection, cells were counted in several parallel cultures, and inoculum was prepared to secure an input multiplicity of infection (MI) of 50-100 LD₅₀ per cell. Adsorption proceeded for 90 minutes at 37° C. After infection, the cultures were three times rinsed and supplied with medium. Because in the system and at the intervals used the virus and IF in the medium represents actually the total IF and TE virus (Libíková and Henslová, 1969), only the medium was used for assay of both in the present experiments. Specimens for assay were kept at -70° C. In experiments done in tube cultures, pools from 3 tubes were used for assay.

Virus assay. TE virus titres were determined by intracerebral inoculation of mice, using 4-6 animals for each virus dilution; the titres were expressed in terms of LD₅₀/ml. WEE virus was assayed in CEC cultures and the titres were expressed in CPD₅₀/ml values.

Interferon assay was performed in CEC cultures with WEE as challenging virus (Vilček, 1961). In specimens for IF assay, TE virus was inactivated by heating at 60° C for 60 minutes.

Immunofluorescence. The indirect technique, using hyperimmune guinea pig antiserum against TE virus and pig anti-guinea pig immunoglobulin conjugated with fluorescein isothiocyanate, was employed. Anti-TE virus serum and the conjugate were absorbed with CEC suspension to prevent non-specific reactions. Coverslip cultures of infected cells were rinsed with saline, fixed for 10 minutes with acetone, treated for 30 minutes with antiserum, rinsed 3 times, treated for 30 minutes with the conjugate at room temperature and after 3 rinses with saline mounted in neutral glycerol. A Reichert fluorescent microscope (ocular $\times 8$, objective $\times 40$, mercury lamp HBO 200) was used.

Results

Altered reaction of old RIF-free CEC cultures to the infection with TE virus as compared with young cultures

Five independent experiments were carried out in tube cultures of RIF-free CEC, 1 and 7 days old. Each cell suspension was prepared from a single embryo. IF titres and virus yields per cell were determined at intervals of 20 minutes (T₀) and 10, 24 and 48 hours after the end of virus adsorption. In all these experiments (Table 1), old cultures yielded more IF and less

Table 1. Interferon and TE virus production in young and old CEC monolayers from RIF-free eggs (input MI = 50–100 LD₅₀ per cell)

Exp. No.	In vitro age of CEC cultures (days)	IF units per ml at the indicated hours after infection				TE virus yield per cell (LD ₅₀)
		0	10	24	48	
1	1	<2	<2	64	64	250
	7	<2	16	1024	512	2.5
2	1	<2	<2	64	64	10
	7	<2	32	512	512	0.1
3	1	<2	<2	16	64	300
	7	<2	32	256	1024	100
4	1	<2	—	<2	2	10
	7	<2	—	—	8	5
5	1	<2	—	<2	<2	5
	7	<2	—	32	16	0.2

Exp. 1–3: Eggs from the Institute of Experimental Biology and Genetics, Prague.

Exp. 4–5: Eggs from the Institut Gustave Roussy, Villejuif.

virus than young ones. CEC prepared from embryos of French origin showed a lower IF production (2 experiments).

Effect of cell density on IF synthesis in one day old cultures

Roux bottles (300-ml) were seeded with 35 ml of CEC suspension, prepared from ordinary embryos. In experiment I, the suspensions contained 5×10^5 or 5×10^6 cells/ml, in experiment II 8×10^5 , 2×10^6 and 3×10^6 cells/ml. After 24 hours of incubation at 37° C, cells in two parallel cultures for each cell inoculum were counted. Based on these counts, the cultures were infected with an input MI of 50 LD₅₀/cell. After 3 days of incubation at 37° C, IF titres were determined in pools from 2 parallel cultures. IF production per 10⁵ cells was only slightly higher in the tenfold denser cultures (Table 2).

Table 2. Interferon formation in TE virus-infected 1-day old monolayers with different density of CEC

Exp. No.	Approx. number of CEC in the monolayer (millions)	Yield of IF 3 days after infection (units)	IF units formed by 10 ⁵ cells
I	3	35	1.2
	39	560	1.4
II	3	0	0
	14	70	0.5
	30	140	0.5

The experiments were carried out in Roux bottles.

Effect of the age of CEC cultures after transfer into suspension cultures

Primary CEC cultures were grown in Roux bottles for 1 or 7 days. Cells were harvested with the aid of trypsin. Portions of $2.5 - 6.0 \times 10^7$ cells in 1—2 ml of medium were inoculated with 1 ml of virus suspension to

Table 3. Production in interferon and TE virus in suspension cultures prepared from 1 and 7 days old CEC monolayers (input MI = 50—100 LD₅₀ per cell)

Hours after virus adsorption	Exp. No.	IF units produced by 2×10^6 cells		Virus produced by 2×10^6 cells (log LD ₅₀)		Yield of virus per cell (LD ₅₀)	
		Age of cells in days					
		1	7	1	7	1	7
0	I	0	0	4.8	4.8	—	
	II	0	0	4.1	4.1		
	III	0	0	4.5	4.5		
24	I	0	64	7.2	6.0	8	0.5
	II	0	4	7.0	5.6	5	0.2
	III	3	64	7.0	4.0	5	—
48	I	0	64	7.8	7.0	30	5
	II	2	16	8.0	5.8	50	0.3
	III	11	64	7.2	6.5	8	1.5

Cultures of ordinary CEC (exp. I and II) or RIF-free CEC (exp. III) were used.

secure an input MI of 50—100 LD₅₀/cell. Adsorption proceeded at 37° C; the suspensions were shaken at intervals. The cells were 3 times rinsed with medium and diluted to make up 20-ml portions containing $4 - 6 \times 10^7$ cells. Suspension cultures were maintained at 37° C. Virus and IF titres were determined at intervals of 20 minutes, and 24 and 48 hours after the end of adsorption. Suspensions prepared from old cultures produced more IF and less virus than suspensions of young cells (Table 3).

Table 4. Detection of TE viral antigen in young and old CEC monolayers by immunofluorescence

CEC from	MI (LD ₅₀ per cell)	CEC cultures infected after					
		1-day cultivation			7-day cultivation		
		A	B	C	A	B	C
Ordinary eggs	100	0.5	3000	44	10.3	150	22
RIF-free eggs	20	16	250	26	256	2.5	13

A: IF units produced by 10^5 cells.

B: Yield of TE virus (in LD₅₀ per cell).

C: Number of fluorescing cells in one field of view determined in tube cultures with 2×10^5 cells (average from 10—50 fields of view).

A, B and C determined 30 hours after infection.

Immunofluorescence in young and old cultures

Coverslip cultures were grown in tubes; other experimental conditions were the same as described above. Cultures infected after 1 day of growth showed immunofluorescence in nearly all cells (Fig. 1), whereas in 7 days old cultures only 20—50% of cells showed positive immunofluorescence (Fig. 2).

In two experiments we compared ordinary and RIF-free CEC cultures (Table 4) with respect to the production of virus (per cell) and IF (per 10^5 cells) and to the counts of immunofluorescence-positive cells. The results were again consistent with our previous findings.

Discussion

Experiments on CEC prepared from RIF-free eggs from two different sources ruled out the possibility that the viruses of the avian leukosis group could be responsible for altered TE virus and IF production in *in vitro* aged cultures. CEC prepared from embryos obtained from Villejuif produced considerably less IF than those from embryos of Prague origin (Table 1). This possibly could be due to the smaller size of 10 days old embryos from Villejuif.

Carver and Marcus (1967) explained the higher IF production in old cultures by a lower level of the repressor of IF production: in contact-inhibited cells macromolecular synthesis is generally decreased.

Contact inhibition in cell cultures should occur when the monolayer becomes confluent. After seeding the cultures with usual cell concentrations (see Methods), 24 hours old cultures are not confluent. Therefore 10 times more concentrated cell suspensions which produced after 24 hours dense, confluent monolayers, were used (Table 2). But in these cultures IF production (per 10^5 cells) was only insignificantly increased. The confluency of the cultures alone, therefore, could not have been responsible for the phenomenon under consideration.

In another experiment we employed cells grown in monolayers for 1 or 7 days, harvested by trypsin, infected and then grown in suspension cultures. Production of IF and of TE virus was determined by the age of cultures before transfer to suspension cultures, even if the contact inhibition was interrupted. On the other hand, Carver and Marcus (1967) have described recovery of high plaquing efficiency of Sindbis virus when cells from old monolayers were trypsinized and grown in subcultures in new monolayers. The difference between their findings and ours is likely to be explained by further cell divisions occurring in the cultures grown by Carver and Marcus. Our subcultures were suspension cultures, in which the cells did not multiply.

Immunofluorescence revealed viral antigen in a smaller proportion of cells in old cultures as compared with young cultures. Intensity of fluorescence in positive cells in old cultures, however, was as strong as in young cultures. At present, we cannot decide whether the cells negative for viral antigen in this test produced infectious virus or not. Nor have we evidence which cells were IF producers.

CEC suspensions were prepared from muscles and skin of chick embryos. They consisted, therefore, of different types of cell; during *in vitro* cultivation, some of them may go through further steps of differentiation (Tikhomirova *et al.*, 1967). This could explain the variability between individual cells in their reaction to viral infection. It is also known that in old cultures some cells divide, whereas others die and thus the total cell count remains constant. These dividing cells can be more susceptible to viral infection. Using a porcine kidney cell line, Korych (1969) was able to detect TE viral antigen by immunofluorescence first in cells which have just undergone mitosis. Some of our preparations from old cultures showed two positive adjacent cells, with surrounding cells negative. This again is consistent with the assumption that dividing cells better support the synthesis of viral antigen.

References

- Carver, D. H., and Marcus, P. I. (1967): Enhanced interferon production from chick embryo cells aged *in vitro*. *Virology*, **32**, 247.
- Henslová, E., and Libíková, H. (1966): Optimal conditions for interferon formation in chick embryo cell cultures infected with tick-borne encephalitis virus. *Acta virol.*, **10**, 475.
- Korych, B. (1969): Biological properties of the tick-borne encephalitis virus (in Czech). Thesis. Institute of Medical Microbiology and Immunology, Charles University, Prague.
- Libíková, H. and Henslová, E. (1969): The correlation between tick-borne encephalitis virus yield and interferon production and effect in cell cultures of different *in vitro* age. *Acta virol.*, **13**, 16.
- Tikhomirova, T. I., Shestopalova, N. M., Amchenkova, A. M. and Karpovich L. G. (1967): Morphological study of cultured cells from trypsinized chick embryo tissues, uninfected and infected with tick-borne encephalitis virus. *Acta virol.*, **11**, 34.
- Vilček, J. (1961): Studies on an interferon from tick-borne encephalitis virus-infected cells (IF). I. Appearance of interferon in infected chick embryo cell cultures. *Acta virol.*, **5**, 278.