Acta virol. 13 : 16–25, 1969

THE CORRELATION BETWEEN TICK-BORNE ENCEPHALITIS VIRUS YIELD AND INTERFERON PRODUCTION AND EFFECT IN CELL CULTURES OF DIFFERENT IN VITRO AGE

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Received July 1, 1968

Summary. — Chick embryo cell (CEC) monolayers cultivated in vitro for 1 or 6 days were infected with tick-borne encephalitis virus (TEV) at an input multiplicity of infection MI = 50 or 0.1 ID_{50} per cell. Virus growth curves and the dynamics of the production of endogenous interferon were determined. At high MI, young CEC cultures produced 48 hours after infection at least 100–1000 ID_{50} of virus per cell, whereas old cultures produced only 0.1–0.2 ID_{50}/cell. The in vitro age of the cultures had the contrary effect on interferon production: young cultures produced 0–3, old ones 8–20 units per 10^5 cells. Low MI resulted in decreased virus yield both in young and old cultures. From these results, certain practical conclusions were drawn.

The earlier finding of an unresponsiveness of TEV-infected CEC to the effect of exogenous interferon (detected by challenge infection with western equine encephalomyelitis — WEE-virus), demonstrable already in the early latent period, was confirmed in the present study also in cultures infected after 6 days of in vitro growth. In this connection the possible role of TEV RNA-polymerase in the reproduction of the challenging WEE virus is discussed.

Introduction

Experiments aimed at obtaining interferon (IF) preparations of high activities revealed that CEC infected with TEV after 6 or 7 days of in vitro growth produce considerably more interferon than one day old CEC (Henslová and Libíková, 1966). Increased IF production is accompanied by decreased virus yield in old cultures as compared to young ones (Libíková and Henslová, 1966). In the present study we attempted to investigate this phenomenon in more detail, by determining growth curves of intra- and extracellular virus (ICV and ECV) as well as of endogenous IF (enIF), i.e. of IF produced in the respective infected culture. Again, titres of intra- and extracellular IF were determined. These findings were compared with the unresponsiveness of TEV-infected CEC cultures to the action of exogenous IF (exIF) (= IF produced in other cultures and added to test cultures) (Libíková, 1965).

Materials and Methods

Cell cultures. CEC suspension was prepared by trypsinization of 10 days old embryos. Tube cultures were seeded with 0.8—1.0 × 10^6 cells in 1 ml of synthetic medium (Slonim *et al.*, 1960) containing 5—10% of heated calf serum. Before infection, the cultures were grown at 37° C for 1 to 6 days; perfect condition of each culture was checked microscopically.

Viruses. The Hy-M line of the Hypr strain of TEV which had undergone 50 intracerebral passages in mice (Libíková and Stanček, 1965) was employed. As stock of WEE virus (strain 15, collection of this Institute) served the fluid from infected CEC cultures, kept at —20° C.

Infectivity assay. TEV infectivity was determined by interference assay using WEE as challenging virus (Vilček, 1960); infectivity was expressed in terms of ID_{50}, a concentration inducing interference in 50% of CEC cultures. Specimens with higher IF activity were titrated in parallel by intracerebral inoculation of mice and LD_{50} values were determined. No significant differences were found between these two techniques of assay. WEE virus infectivity was determined in CEC cultures according to the cytopathic effect (CPE) scored three days after infection; the titres were expressed in CPD_{50} values. In experiments, in which WEE virus was titrated in CEC cultures at different intervals after their infection with TEV, WEE virus dilutions were added to the cultures without change of the medium.

Each virus dilution was inoculated into 2 or 4 tube cultures of CEC (0.1 ml per tube) or 4 mice.

Interferon. Six days old CEC cultures in Roux bottles were infected at high MI with Hy-M virus and medium from them was harvested 3 days later. IF was partially purified by precipitation with acetone (Žemla and Vilček, 1961). This preparation was employed as exIF.

Interferon assay. IF titration was performed in 1 day old CEC cultures, using WEE virus according to Vilček (1961): growth medium in test cultures was substituted by 1 ml volumes of twofold IF dilutions (1 : 2—1 : 1024) in maintenance medium; simultaneously each culture was infected with 100 CPD_{50} of WEE virus in 0.1 ml. Each IF dilution was added to 2 or 4 cultures. The CPE was scored after 3 days of incubation at 37° C. IF titres were expressed in terms of units per 1 ml or per 10^5 cells.

Unresponsiveness to IF of TEV-infected cultures was determined in a similar manner; it was expressed in terms of the titre of exIF and compared to uninfected cultures.

Infection of CEC cultures with Hy-M TEV. The amount of the cells was determined as an average from 3 tube cultures; it was usually 1.5—4.0 × 10^5 cells, old cultures were usually somewhat denser. Growth medium from the cultures was removed and virus was added in 0.1 ml volumes to the cultures; the virus dilutions used corresponded to MI = 50 or 0.1 ID_{50}/cell, respectively. Adsorption proceeded for 90 minutes at 37° C; thereafter the cultures were rinsed four times and supplied with 1 ml of fresh medium. Further incubation proceeded again at 37° C.

Virus and enIF growth curves. At given intervals, always three tube cultures were withdrawn and treated as follows. Medium from all three cultures was pooled and kept at —70° C. Cell monolayers were 4 × rinsed with medium, 1 ml portions of medium were added and the cells were disintegrated by three cycles of freezing and thawing, using dry ice with ethanol. The contents of 3 tubes were again pooled and kept at —70° C. Virus and enIF assay was performed 1—4 days later. The materials were divided into two parts: one was used for assay of ICV and ECV (total virus was calculated as the sum of ICV + ECV); the second part was heated for 1 hour at 56° C and used for ICIF and ECIF determination.

Arrangement of the experiments. The required amount of 1 or 6 days old cultures was infected with TEV. At intervals of 2, 5, 10, 17, 24, and 48 hours after the end of adsorption, specimens were withdrawn for determination of ICV, ECV, ICIF and ECIF. At the same intervals, determinations of unresponsiveness to exIF and of sensitivity to WEE (by titration of WEE in the cultures) were carried out. In parallel, control titrations of exIF and of WEE virus were performed in uninfected 1 and 6 days old CEC cultures.

Both 1 and 6 days old cultures were always set up from the same CEC batch. Cultures to be 6 days old at the time of infection were seeded first. CEC suspension for young cultures was kept at 4° C and the cultures were set up 5 days later; both sets of 1 and 6 days old cultures were infected at the same time.

Results

Experiments in 1 day old cultures

Two of several growth curves in young cultures infected at high MI are represented in Fig. 1. The yield of virus was high, whereas up to 48 hours no enIF production could be detected by the method used. In some of the

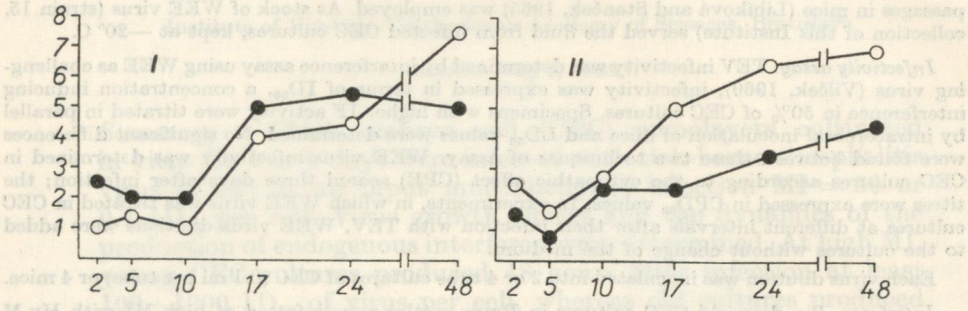

Fig. 1.

Growth curve of TEV in CEC monolayers infected after one day of in vitro growth
I and II — two independent experiments, MI = approx. 50 ID_{50} per cell.
Ordinate: log ID_{50} of virus (● ICV, ○ ECV) in one tube.
Abscissa: time of incubation in hours after the end of adsorption period.
Neither ECIF nor ICIF was detected during the course of the experiments.

other experiments, threshold activity of IF was found at the last intervals.

At low MI, virus yields were lower and, at 48 hours after infection, low levels of enIF could be detected.

Experiments in 6 days old cultures

Two experiments, parallel to those shown in Fig. 1, are illustrated in Fig. 2. Except of the age of cultures, all other conditions were the same. Virus yield

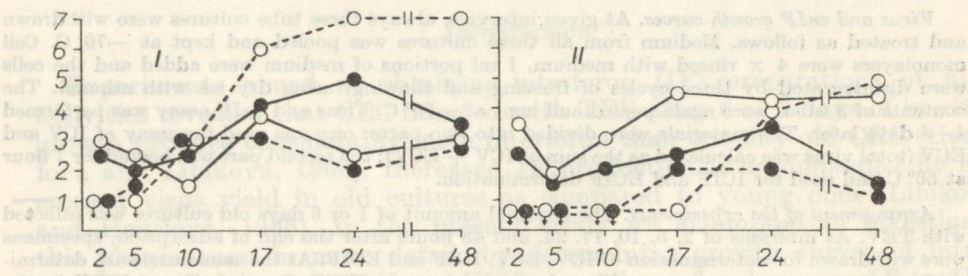

Fig. 2.

Growth curves of TEV in CEC monolayers infected after 6 days of in vitro growth
I and II — two independent experiments, MI = approx. 50 ID_{50} per cell.
Ordinate: ID_{50} of virus (———) per tube (log_{10}) or titres of enIF (- - - - -) (log_2).
Abscissa: hours of incubation after the end of adsorption period.
● ICV or ICIF; ○ ECV or ECIF.

was lower, but there was no shift in the latent period. The production of enIF was considerably different from 1 day old cultures. In exp. 1, interfering activity was detected in intracellular material 5 hours after infection; IF activity in the medium was detected after 10 hours. In exp. 2, ICIF was

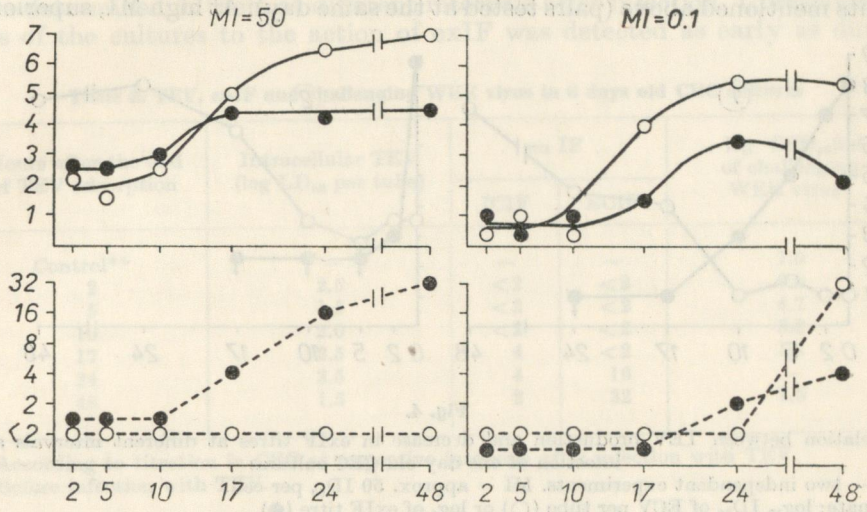

Fig. 3.

Comparison of virus and interferon production in CEC monolayers of different in vitro age; MI approx. 50 or 0.1 ID_{50} per cell.
Upper part: total virus growth curves (ordinate = log ID_{50} per tube).
Lower part: interferon growth curves (ICIF and ECIF, ordinate: IF titres).
○ 1 day old cultures; ● 6 days old cultures.

first detected 17 hours after infection, ECIF was found at 24 hours. Two days after infection, the titre of ECIF in exp. 1 and 2 was 128 and 32, respectively.

In 6-day cultures, low MI resulted in a markedly decreased production of both virus and IF.

Table 1. Production of TEV and IF in CEC cultures depending on the age of cultures and on MI

Age of cultures (days) at the time of infection	High MI (50 ID_{50}/cell)		Low MI (0.1 ID_{50}/cell)	
	ID_{50}	IF	ID_{50}	IF
1	100—1000*	0—3*	3	1
6	0.1—0.2*	8—20*	0.01	1.3

ID_{50} = virus yield/cell at 48 hours after infection.
IF = interferon yield (units/10^5 cells) at 48 hours after infection.
* Based on several experiments.

Relationship between MI, virus and IF production, and in vitro age of CEC cultures

The relationships between the age of cultures, MI and virus and IF yields are evident from growth curves in Fig. 3. The curves were based on the experiments mentioned above (pairs tested at the same day). At high MI, superiority

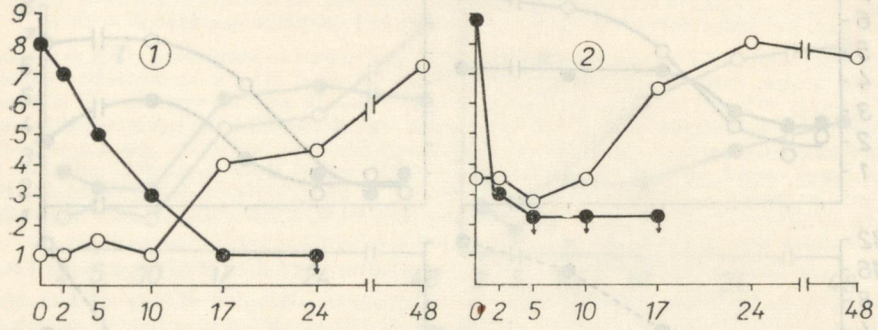

Fig. 4.

Correlation between TEV production and decrease in exIF titres at different intervals after infection of one day old CEC cultures
1, 2 — two independent experiments. MI = approx. 50 ID_{50} per cell.
Ordinate: $\log_{10}$ ID_{50} of ECV per tube (○) or $\log_2$ of exIF titre (●).
Abscissa: hours of incubation after the end of adsorption period.

of young cultures for virus production and superiority of old cultures for IF production becomes evident. At low MI in young cultures virus production was depressed and IF production enhanced. In old cultures at low MI, both virus and IF production were lower.

These relationships become still more marked, when virus yield in 1 and 6 days' cultures is expressed in terms of LD_{50}/cell and enIF production in terms of units/10^5 cells (Table 1).

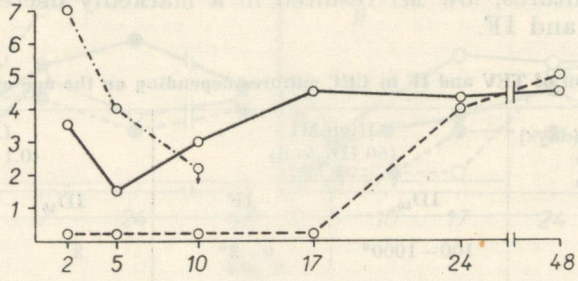

Fig. 5.

Decrease of the effect of exIF in 6 days old cultures infected with TEV
Ordinate: $\log_{10}$ ID_{50} of ECV per tube (———); $\log_2$ of enIF titres (- - - -); or $\log_2$ of exIF titres (— . — . —).
Abscissa: hours after the end of adsorption period.

Unresponsiveness to the action of exIF of CEC cultures infected with TEV at different intervals after seeding

Two out of 6 independent experiments are illustrated in Fig. 4. One day old cultures were infected at high MI with TEV and the activities of exIF were determined in them at different intervals after infection. Unresponsiveness of the cultures to the action of exIF was detected as early as during

Table 2. TEV, enIF and challenging WEE virus in 6 days old CEC cultures

Hours after the end of TEV adsorption	Intracellular TEV (log LD_{50} per tube)	en IF		log CPD_{50}/ml of challenging WEE virus*
		ICIF	ECIF	
Control**	—	—	—	7.0
2	2.5	<2	<2	6.0
5	1.5	<2	<2	4.7
10	2.0	<2	<2	3.2
17	2.5	4	<2	1.7
24	3.5	4	16	—
48	1.5	2	32	1.5

* According to titration in CEC at respective intervals after infection with TEV.
** Before infection with TEV.

the latent period which is consistent with our previous findings. No activity of the added exIF could be detected 17 and 5 hours after the end of adsorption in exp. 1 and 2, respectively. In these cultures, no production of enIF was found until the end of the experiment.

We were interested to see whether this unresponsiveness would develop also in 6 days old cultures. Here, however, an early production of enIF had to be considered. Fig. 5 shows the result of an experiment which demonstrates that the exIF exerts a decreased activity also in 6 days' cultures infected with TEV. In this case the challenging dose of WEE virus was 10^5 CPD_{50} per culture. In the control and 2 hours after infection with TEV, the exIF

Table 3. TEV, enIF and challenging WEE virus in 1 day old CEC cultures

Hours after the end of TEV adsorption	Intracellular TEV (log LD_{50}/tube)	enIF		log CPD_{50}/ml of challenging WEE
		ICIF	ECIF	
Control	—	—	—	8.5
2	3.0			8.0
5	1.0			7.5
10	2.5			7.0
17	3.5	>2	>2	8.5
24	5.0			7.5
48	5.5			8.0

For explanations see Table 2.

showed a titre of 128, at 5 hours it decreased to 16 and at 10 hours to less than 4. In this experiment enIF appeared first 17 hours after infection with TEV and then protected the cultured from the CPE of challenging WEE virus, so that exIF could no longer be demonstrated. This experiment clearly showed that in 6 days old cultures exIF could not fully express its action during the first 10 hours after infection with TEV, whereas in the same set of cultures enIF was able to protect the cells from the cytopathic activity of WEE virus. This follows from data presented in Table 2, illustrating the results of WEE titrations in CEC cultures at different intervals after infection with TEV (the experiment was performed in parallel with that in Fig. 5). At 17 hours after infection with TEV, enIF was able to inhibit more than 10^5 CPD_{50} of challenging WEE virus. An eventual objection that TEV could interfere with WEE virus also by some non-interferon mechanism is refuted by experimental data in Table 3 on 1 day old CEC cultures: the titre of superinfecting virus did not decrease, as at the same time no enIF could be detected.

Discussion

Our experiments on interferon production in old CEC cultures were inspired by the occasional finding of autoinhibition zones (without a CPE) in titrations of WEE virus stocks which had been produced in older tissue cultures. The presence of IF in virus stocks could have accounted for the inhibition zones. Having detected higher IF levels in older CEC cultures infected with TEV (Henslová and Libíková, 1966), we started to search the literature for similar data.

The effect of in vitro age of chick embryo fragments on the production of herpes simplex virus was investigated by Cheever and Wilmert (1942; cit. after Frothingham, 1959). Virus did not multiply in cultures more than four days old. In human amnion cultures infected with RMC virus the inhibitory activity against poliovirus was higher in cultures 19—35 days old than in 13 days old or younger cultures (Ho and Enders, 1959). Our findings on TEV-infected cultures of HeLa cells of various age were consistent with these data (Libíková and Smidová, 1961). The CPE caused by TEV decreased with the age of cultures. In some cases, however, sensitivity of cell cultures to viral infection increased with the age. Newcastle disease virus reached higher titres and produced a more marked CPE in 6—8 days old cultures than in younger cultures (Kumagai *et al.*, 1958). Also poliovirus and Sindbis virus exerted a higher cytopathic activity in human amnion cultures 30 days old or older than in 5—10 days old cultures (Frothingham, 1959).

In primary CEC cultures, Sindbis virus induced a higher IF production after 7 days of in vitro growth than in younger cultures. In older cultures the efficiency of plating and virus reproduction were also considerably lower (Carver and Marcus, 1967). In the case of Newcastle disease virus, which is known to be little sensitive to IF, age of the cultures had no significant effect. The authors assume that higher IF production in older cultures might be due to a lower concentration of cellular repressors, so that the latter

can be more readily inactivated by virus-derived inducers or de-repressors. An effect of contact inhibition in old cultures could also play a role here.

The present results are essentially consistent with considerations of Carver and Marcus (1967), with the reservation that at low MI not only the production of virus, but also that of IF is reduced. Our further experiments are aimed at separating the effects of the age and of contact inhibition.

Although there remains much to be done for the elucidation of the mechanism of increased IF and decreased virus production in old cultures, already from the present results certain conclusions for practical virology can be made. When primary cell cultures are to be used for virus isolation, quite young cultures are certainly superior. The same applies also for the production of virus vaccines, where high virus titres are required. This conclusion, reached also by Gagarina (personal communication), is of importance in the production of TEV vaccine in CEC cultures. On the other hand, for IF production older cultures are superior. Less unequivocal is the determination of the suitable age of culture for the titration of noncytopathic viruses by interference with a challenging cytopathic virus. Cultures more than 3 days old are not suitable for infection by the noncytopathic virus, because in such cultures, after a small inoculum, de novo virus production is lower, and this could adversely influence the titres determined. It is important to challenge the cultures not earlier than 3 days after inoculation with the virus to be titrated, so that sufficient IF levels be reached.

Finally, there remains the question of unresponsiveness to exIF in TEV-infected cells (Libíková, 1965; Stanček, 1966). This unresponsiveness becomes expressed early after infection of the cells with TEV and it does not result from persistent infection as has been originally assumed from experiments on L cells (Vilček and Stanček, 1963).

Somewhat similar phenomena were observed by several authors working with different systems and under different conditions (Cantell and Valle, 1965; Ghendon 1965; Chany and Brailovsky, 1965; and others). These findings may signalize either some helper effect between different viruses, or stimulons of viral reproduction, or perhaps some inhibitors of IF in infected cultures.

We have attempted to elaborate a working hypothesis to explain the unresponsiveness of cells to exIF, which results from their infection by TEV. One possibility is that TEV infection prevents the synthesis of antiviral protein following after IF treatment. But this hypothesis can hardly explain the case in which TEV-infected cultures first showed unresponsiveness to exIF, but later were protected against WEE virus by enIF, which most likely had induced the antiviral protein. Another alternative is a possible cooperation between TEV and WEE virus, present in the given system. Both of them are small enveloped RNA viruses. In Semliki virus (which also is an arbo A virus, like WEE virus) it has been assumed that IF prevents the synthesis of its RNA-polymerase (Sonnabend *et al.*, 1967). It is not unlikely that IF could act similarly on WEE virus. In our system, however, both IF and WEE virus were added to cell cultures, where TEV already started to repplicate and had already synthesized its RNA-polymerase. It has been established that in some

instances RNA-polymerase is able to replicate RNA of other, usually related viruses. Such is the case of guanidine-sensitive and guanidine-resistant variants of poliovirus (Wecker and Lederhilger, 1964), of different types of poliovirus (Agol and Shirman, 1965), or of MS-2 phage and turnip yellow mosaic virus (Haruna *et al.*, 1963). Similarly, we consider it possible that WEE virus could utilize the TEV-induced RNA-polymerase in the case that exIF prevents the synthesis of its own RNA-polymerase. In such TEV-infected CEC cultures, to which exIF had not been added, enIF at a later interval might exert its effect, because the activity of RNA-polymerase may be gradually lost. It is difficult at present to decide how and if at all also polysomes induced by TEV infection might be involved (e.g. Martin, 1967).

Acknowledgements. We appreciate the useful discussions with Dr. J. Závada and an expert technical assistance of Mrs. V. Kostolanská and Mrs. A. Styková.

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