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BEHAVIOURAL PATTERNS OF TICK-BORNE ENCEPHALITIS VIRUS VARIANTS IN HUMAN NERVOUS CELLS IN VITRO

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Summary. — A variants of tick-borne encephalitis (TE) virus highly neurovirulent for *Macaca mulatta* monkeys multiplied well and exerted a pronounced cytopathic effect in cells originating from human embryonic cerebellar tissue cultivated in vitro. The capacity of the virus variant attenuated for monkeys to propagate in these cells was extremely restricted and it was possible to maintain the virus only in the first passages. The findings in this model system are discussed in relation to the phenomenon of the virulence of TE virus.

Introduction

Although the in vitro cultivation of TE virus has been studied in a considerable extent and for various purposes in the last decade, there are practically no data concerning the interaction of this virus with human nervous cells. The damaging effect of TE virus or of the closely related louping-ill virus on nervous cells in vivo is well known from the description of pathological changes resulting from the natural or experimental infection (see review by Zilber, 1962). In spite of the recent development and refinements of the methods enabling the cultivation of human central nervous system cells and the known high susceptibility to TE virus of nervous tissue in vivo, their mutual in vitro relationships have not yet been described. This kind of human tissue, however, appeared to be suitable for investigation of the behavioural patterns of two TE virus variants, differing substantially in their neurovirulence for monkeys (Mayer, 1966; Mayer and Rajčáni, 1967).

This model system, with all limitations resulting from its in vitro nature, could nevertheless bring certain valuable informations concerning the capacity to propagate in known human nervous tissue of a definite TE virus clone possessing extremely limited ability to multiply in monkey brain.

Materials and Methods

Virus. The P III-E clone, highly virulent for monkeys, in its 4th mouse intracerebral (ic) passage after cloning, and the attenuated Hy-HK28"2" clone in its 2nd mouse ic passage, both derived (Mayer, 1966) from the Czechoslovak "Hypr" strain, the prototype of the western subtype of TE virus (Taylor, 1967), were used. The titre of the 10% infected mouse brain suspension was 9.2 log ic LD_{50}/ml in the case of the P III-E virus and 8.0 in the case of the Hy-HK28"2" virus.

The extracellular virus was assayed in 6—8 g mice by ic inoculation of tenfold diluted culture fluids and its amounts expressed in log LD_{50}/ml values.

Tissue cultures. Human cerebellar and retinal tissues were used. The embryos obtained by artificial abortion were on the average 10 weeks old. From 3—4 explants were embedded by the plasma clot technique on coverslips and then maintained in test tubes with 1 ml of nutrient medium consisting of 20% heated calf serum in synthetic medium 199 (with 6 mg/ml of glucose) and 5% of chick embryo extract. In some experiments, Eagle's basal medium instead of medium 199 was used. The tissue explants were cultivated in a roller tubes system and refed with fresh medium each 3rd day.

Culture tubes in which good outgrowth zones were observed, usually after 12 days of cultivation, were chosen for virus infection. After removing the nutrient medium, 0.1 ml of diluted suspension, containing approximately 10^5 ic LD_{50} of virus, was dropped on the coverslip with explants and allowed to adsorb for 90 minutes at 37° C. After repeated washing of the coverslips, fresh medium was added to the cultures and the tubes incubated again in the roller system. From 4—10 tubes (i.e. 14—35 explants on the average) were infected for each virus passage or experiment.

The control cultures were inoculated with an appropriately diluted normal brain suspension. Some controls consisted of virus-inoculated cultures, but without renewal of nutrient medium at the time of infection.

The rate of TE virus multiplication was investigated in parallel cultures of the human embryonal retina and cerebellar tissue originating from the same embryos.

Results

The propagation of TE virus

In a series of experiments on both cerebellar and retinal tissue cultures the rate of multiplication of the virus clones used was followed in 5 successive passages. The virus was allowed to multiply in the cultures for 5 days. The samples of culture fluid from individual tubes were pooled on the 3rd and 5th day after inoculation and titrated in mice (extracellular virus). The undiluted harvests from the 5th day served also as inoculum for the next passage.

The P III-E virus multiplied well during the serial passages; 4—6 log LD_{50} of virus were detected in cerebellar and 3—5 log LD_{50} in retinal cultures at the end of each of the five successive passages. Hy-HK28"2" virus multiplied to a limited extent, with virus yields decreasing progressively from passage to passage. However, no virus was detected in cultures inoculated with the attenuated virus starting from the fourth passage (Table 1). Similar events were observed in experiments on both retinal tissue and cerebellar explants.

In control experiments, roller tubes with medium only were inoculated in a similar way as the tissue cultures to clarify the influence of the initial virus inoculum and that of its thermal inactivation on the virus titres observed in the experiments described. Under such conditions, the active P III-E virus was detected only on the 5th day and the Hy-HK28"2" on the 3rd day of their first in vitro passage. Thus it was clear that the initial virus inoculum did not influence the results obtained.

When either virus was neutralized with immune serum, and the cultures inoculated with the serum-virus mixtures, virus multiplication was not observed.

Table 1. Propagation of tick-borne encephalitis virus variants in human nervous tissue roller cultures

Human embryonic tissue system	Virus clone inoculated (inoculum: 10^5 ic LD_{50})	Titre (log ic mouse LD_{50}/0.03 ml) at passage level									
		I		II		III		IV		V	
		3*	5*	3	5	3	5	3	5	3	5
Cerebellum	P III-E	5.5	ND	6.5	6.8	4.5	4.2	3.7	4.5	6.0	5.0
	Hy-HK28"2"	3.2	ND	3.2	1.5	1.2	1.2	< 0.5	< 0.5	< 0.5	< 0.5
Retina	P III-E	6.5	4.5	4.5	5.2	ND	3.7	3.7	3.5	4.5	4.7
	Hy-HK28"2"	4.2	3.5	2.5	2.5	ND	2.5	< 0.5	< 0.5	< 0.5	< 0.5

* 3 and 5: titres determined on days 3 and 5, respectively.
ND = not done.

Cytomorphological observations

The first migration of the cells from the maternal cerebellar explants was observed on the 3rd day of cultivation. The outgrowth zone appeared on several sites of the explants and in variable extent.

Among the migrating cells it was possible to differentiate the cells of mesenchymal origin and ependymal and neuroglial cells. The spindle-shaped mesenchymal cells, together with the glial cells, formed network-like structures in the outgrowth zone (Figs 1—3). On its edge, in the cell-free plasma clot, we observed large flattened mesenchymal cells, star-shaped cells with numerous processes and large multinucleate cells described by Hild (1957*a*) and Costero and Pomerat (1951).

The cells of ependymal origin were observed only in the outgrowth zone. They formed membranaceous structures with typical rosettes (Hild, 1957*b*; Hogue, 1947). In some cultures, the whole outgrowth zone consisted of cells with dark nuclei and a narrow cytoplasmic zone forming also membranes. Their outgrowth resembled very closely the outgrowth of "matrix cells" (Miyake *et al.*, 1961) from which the neuroblasts and spongioblasts differentiate.

On the 5th day after inoculation, the infected cultures were fixed in Carnoy's fluid or in 10% formol in Gey's solution. They were stained with haematoxylin-eosin, toluidin or anilin blue, and cresylviolet.

As controls we employed uninfected cultures and also infected and uninfected cultures cultivated under less favourable conditions, i.e. without renewal of medium, in order to ascertain the role of possible nonspecific degeneration in the incidence of cultures cells.

For cytomorphological study we used only cultures from passage I—III in which both P III-E and Hy-HK28"2" viruses were shown to multiply. The findings were verified in a separate experiment where groups of 10 cultures each were infected with either virus (i.e. their first tissue culture passage) and the same number of tubes "inoculated" with appropriate dilution of normal mouse brain suspension served as controls.

Marked differences were observed between the behaviour of the monkey-virulent P III-E clone on the one hand of the Hy-HK28"2" virus and controls on the other.

The outgrowth zone in cultures infected with the P III-E virus was almost completely destroyed, with only small remaining areas (Fig. 4).

The cytopathic effect exerted by the monkey-attenuated Hy-HK28"2" virus on the outgrowth zone was far less destructive as seen with the P III-E virus (Fig. 5). The number of mesenchymal, neuroglial and neuroepithelial cells differed only slightly from the controls. The number of large star-shaped cells present was also considerable.

The mitotic indices (estimated on the basis of mitotic activity of 100 nuclei of each explant) showed very marked differences between controls and either of infected cultures (cerebellar and retinal — where considerable part of the outgrowth zone was of mesenchymal origin). The action of the P III-E virus

damaged the mitotic mechanism more seriously than the Hy-HK28"2" virus, as shown by the following data on mitotic indices:

	P III-E	Hy-HK28"2"	Controls
Cerebellar tissue	0.5	1.2	2.4
Retinal tissue	0.55	0.75	1.6

In both infected and uninfected explants maintained under unfavourable conditions, the signs of nonspecific degeneration were observed, but the general appearance (destruction of the outgrowth zone by the P III-E virus) was the same as in the experiments mentioned above (Figs 6—8). The most striking feature differentiating these two kinds of cultures was the observed granulation of all migrated cells and the large number of macrophages present in explants cultivated under suboptimal conditions, which was not the case in the virus-induced destruction. The incomplete cytopathic effect of the P III-E virus, observed in these cultures could be, possibly, in relation to the worsened physiological state of undernutritioned cells.

The specificity of cytopathic changes observed was ascertained with the aid of specific immune serum.

Discussion

The extent of virus multiplication during the serial passages may probably reflect the different capacity of the variants studied to multiply in the human embryonic nervous system. With certain reservation, this observation could find certain parallel in the behaviour of these two TE virus clones after ic infection of monkeys. The low rates of multiplication with gradual decline in infectious titres might support the concept of low susceptibility of primate nervous cells to the infection with the attenuated TE virus used. In preliminary investigations by the fluorescent antibody technique, specific fluorescence was detected in almost all visible cellular elements present in the virulent virus-infected explants and outgrowth zone. In the explants infected with the Hy-HK28"2" virus, the fluorescence was of markedly lower intensity and it was observed only in scattered cells, mainly of the outgrowth zone. The cellular damage was regularly noted in explants infected with the monkey-virulent virus.

The early developmental stage of the explanted material and the relatively short time of cultivation (roller tubes) did not allow a pronounced differentiation of migrated cells. In the very young embryonic cerebellar tissue used for cultivation, the large neuronal cells are localized either in the ependymal or in the subependymal layer (Miale and Sidman, 1961; Miyake *et al.*, 1961; Meyer, 1963; Duckett and Pearse, 1965) in the form of spindle-shaped or star-shaped cells without any specific morphological characteristics. Their differentiation during the two weeks' cultivation in roller tubes cannot be completed (Nissl's substance, neurofibrils, myelin formation) and therefore it was impossible to study the interaction of individual neuronal cells with TE virus. Nevertheless, in the outgrowth zone we observed the cells resembling by their size, shape, the number and character of processes and the size

of nucleus and nuclei, the non-differentiated nervous cells. But we presume that owing to the superficial localization of neuronal cells and their migratory activity, this differentiation could be achieved in long-term cultures.

The present findings on TE virus variants studied resemble to a certain extent those on the H (human) marker of attenuated polioviruses, where with certain strains, e.g. the highly attenuated CHAT strain, a lower affinity for in vitro cultivated human tissue was observed (Gard, 1960), or on the multiplication ability of certain Langat virus lines in some human cell strains (Thind and Price, 1966). It is too early to draw definite conclusions, but the problem as to whether a correlation exists between the grade of multiplication of definite TE virus clones in nervous tissue in vitro and "neurotropism" or "neurovirulence" as revealed clinically in vivo, deserves further consideration. Furthermore, such in vitro observations of interactions between cultivated, more differentiated, nervous cells and viruses, e.g. between human cerebellum cells and TE virus, might offer data more or less relevant to the research on the encephalitogenic potential of TE viruses in man. Such a model may be of certain importance, especially in asituation in which the possibilities of experimentts on the final host are lacking

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Explanations of Photomicrographs

Cerebellum from 9 weeks old human embryo, 14 days in vitro

Fig. 1. Giant cells with one multinucleate cell; cresylviolet.

Fig. 2. Large, star-shaned cell.

Fig. 3. Outgrowth zone in an uninfected explant.

Fig. 4. Destroyed outgrowth zone, 5 days after infection with 10^5 mouse ic LD_{50} of the P III-E clone of TE virus.

Fig. 5. The outgrowth zone, 5 days after infection with 10^5 mouse ic LD_{50} of the Hy-HK28"2" clone of TE virus.

Fig. 6. Outgrowth zone in an uninfected explant, cultivated without renewal of medium; hematoxylin-eosin.

Fig. 7. Destroyed outgrowth zone, 5 days after infection with 10^5 mouse ic LD_{50} of the P III-E clone of TE virus; cultivated without renewal medium.

Fig. 8. Outgrowth zone, 5 days after infection with 10^5 mouse ic LD_{50} of the Hy-HK28"2" clone of TE virus cultivated without renewal of medium.

Magnification: Figs 1 and 2 — approx. × 400, Figs 3—8 — approx. × 100.