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A Study of the Development of the Tick-Borne Encephalitis Virus in Human and Animal Tissue Cultures

by G. D. Zasukhina - the Institute of Virology in name of D. I. Ivanovskiy AMN USSR, Moscow.

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During the past 5-7 years the tissue-culture method has acquired a great significance in virological investigations. Now, the important characteristic of any virus is not only its pathogenicity for laboratory animals, but, also, its pathogenicity for tissues in vitro. It is known from the literature that the tick-borne encephalitis virus propagates readily in chick-embryo tissue cultures according to the Meytlandov method (1-3), in cultures of human embryonic tissues with plasma (4, 5), and in chick-embryo and HeLa cell cultures without plasma (6).

We studied the adaptation, the intensity of growth, and the ability of the tick-borne encephalitis virus to induce a specific cytopathogenic effect in vitro, depending on the type of tissue. In our study of the conditions of the virus's reproduction we used tissues of animals that are highly or slightly susceptible to tick-borne encephalitis; also used were connective-tissue and epithelian elements, both normal and malignant, and also embryonic or postnatal tissues of humans and animals.

The experiments were conducted with the strain "Sof'in," which has been passed on in the laboratory in white mice since 1937.

Plasma cultures were prepared from the skin-muscle, lung and kidney tissues of a human embryo, from the skin-muscle tissue of white mice and rats, and from the tissue of a chick embryo. Monkey and dog kidney tissues were trypsinized. The inoculation of the plasma cultures was made 48-72 hours after the explant; trypsinized cells and HeLa cells were inoculated on the 6th-7th day of growth after a single change of the nutrient medium. The tissue cultures were grown in Ender's medium, or in a medium consisting of bovine amniotic fluid, 40 %, Hank's solution, 40 %, and inactivated bovine serum , 20 %. In the inoculation of the cultures we were using medium No. 41 according t Barski, Ender's medium, and medium No. 199.

By cultivation of the tick-borne encephalitis virus in the various tissue cultures we were able to produce its regular propagation. The passages in the culture containing the skin-muscle tissue of a human embryo were carried out 40 times; in the kidney and lung tissue of the human embryo, in the HeLa cells,

and in the chick-embryo, white-mouse and rat tissues it was carried out 5-7 times; and in the monkey and dog trypsinized cells it was carried out 4 times.

In table 1 are presented the data from the fifth passage's viral titration, by the method of intracerebral inoculation of white mice, in the cultures of the various tissues.

As is evident from the table the virus's maximum titers were observed in all of the tissues during the 3rd-8th day of cultivation. The highest titer of the virus was received in the cultures of skin-muscle tissue of a human embryo and in the HeLa cells (lg LD_{50} 6.3-6.5). The titer of the virus dropped on the eighth day after inoculation, although the virus retained its viability in the tissue cultures relatively long. The virus was detected up to 44 days after inoculation into the cultures of human embryonic skin-muscle tissue without changes of the cultural fluid[1]. The virus was detected in the cultural fluid of the HeLa cells, where the medium was changed every 3-4 days, on the 25th day after inoculation. By the 40th passage the dilution of the original viral inoculum reached 10^{80}; the cumulative titer in lg LD_{50} for the mice in an intracerebral titration amounted to 200.32; the cumulative titer[2] in lg $TTsPD_{50}$[3] was 216.2. These data indicate an intense viral propagation in the tissue culture. The lowest viral titers were in the cultures of chick-embryo lung tissue and white-mouse embryonic skin-muscle tissue (the lg LD_{50} amounted to 3.0-4.0).

An expressed cytopathogenic effect was registered in the cultures of the human embryonic skin-muscle and kidney tissue. There was no cytopathogenic action detected on the human lung tissue <u>in vitro</u>. A cytopathogenic effect was observed in the cultures of human skin-muscle tissue from the first passage. The first indications of cellular destruction could be noted within 1-2 days with a low-power magnification of a microscope. Complete disintegration of the tissue came later, on the 4th-5th day. The incubation period of the cytopathogenic action was decreased to three days, according to the measure of passage. The brain suspension that was used for the passage in the tissue cultures was titrated in a parallel-test inoculation of tissue cultures and white mice. The final results of the experiments in the tissue cultures were read on the 14th day after inoculation, and in the mice - on the 21st day. The viral titers in the tissue cultures and in the mice were approximately the same and amounted to 10^7. Later, we conducted a comparative titration of the cultural fluid of the 12th passage (table 2).

It is evident from table 2 that the viral titers in the tissue cultures somewhat exceeded the titers in the mice, but the pattern of the virus accumulation, with its maximum release into the cultural fluid on the 3rd-8th day, persisted.

After the 28th passage we observed the phenomenon of the virus's autointerference. In the tissue cultures inoculated with the whole cultural fluid the cytopathogenic effect developed at later periods (7th day) and the cells were not completely destroyed (the intensity of degeneration did not exceed + or ++)[4]. In the tissue cultures inoculated with dilutions of the cultural liquid, 1:100 to 1:1,000, an expressed cytopathogenic effect was registered on the third day after inoculation (the intensity of degeneration was +++ or ++++).

In the process of growing the tick-borne encephalitis virus in the human skin-muscle tissue we detected that the virus's cytopathogenic effect was not induced if we used a cultural liquid that had been kept at a temperature below 0°C for the inoculation instead of the native cultural fluid. A 10-% brain suspension that had been prepared on a serum medium lost its cell-disintegrating ability after a two-week retention, although the propagation of the virus continued. Evidently the factors stipulating the destructive effect of the virus on the cells lower or lose their activity at a temperature below 0°C. With the use of a defrosted cultural liquid in the passage the cytopathogenic action of the virus was restored only after 3-5 passages.

The specificity of the virus's cytopathogenic effect was determined by extinguishing it with serum from a patient suffering from tick-borne encephalitis. For this purpose, equal amounts of the cultural fluid from the 23rd passage and from a 10-% brain suspension (1,000 $TTsPD_{50}$) were mixed with equal amounts of serum in dilution of 1:4-1:512. As a control we used mixtures of equal amounts of the viral suspension and human whole serum (Control No. 1), the viral suspension and a serum specifically immune to Japanese encephalitis (Control No. 2), and 1,000 $TTsPD_{50}$ of the virus without serum (Control No. 3). After the mixtures were retained at room temperature for one hour they were then introduced into tissue cultures. The results were read on the 3rd-5th day after inoculation (table 3).

A cytopathogenic effect was registered in all of the test tubes inoculatedwith the mixture of the virus and the normal human serum and the mixture of the virus and the serum immune to Japanese encephalitis, and in the test tubes inoculated with 1,000 TTsPD50 of the virus. The titers of the serum containing the brain and cultural antigen were practically the same. The serum that was taken from the patient on the 5th and 15th day of the disease neutralized the virus in the white mice and in the tissue cultures. Both in the mice and in the tissue cultures an increase of antibodies was noted by the 15th day of the disease. The nutrient medium exerts a great influence on the development of the virus's cytopathogenic effect.

It has been noted in our work (5) that medium No. 199 is not favorable for the development of the tick-borne encephalitis virus's cytopathogenic effect. This is possible due to the less intense reproduction of the virus in the tissue cultures containing this medium (viral titers are 0.5-1.0 lg LD_{50} lower). The presence of a salt solution in the nutrient medium exerts a favorable influence (Hanks, Earle or Tirode). With the use of Ender's medium, which does not contain the salt solution, the cytopathogenic effect comes 1-2 days later and the cellular destruction is not always complete. Reissig, Black and Melnick explain the delay in the cytopathogenic effect by the lack of amino acids, particularly glutamine, as well as by the lack of some salts in the medium. Inactivated horse serum is more favorable than cow serum for developing the cytopathogenic effect of the virus. The titers of cytopathogenicity in the tissue cultures with the horse serum were always higher than those with the cow serum.

In the cultures containing the kidney tissue of a human embryo, disintegration of the fibroblasts was observed with the same regularity as in the

skin-muscle tissue. But the epithelian cells, which comprise 30-50 % of an outgrowth of young cells, were not destroyed by the virus.

The cytopathogenic action of the tick-borne encephalitis virus was noted also in the cultures containing the skin-muscle tissue of a white-rat embryo. Cellular disintegration, however, was not registered in all of the passages. The cytopathogenic effect was absent in the second and sixth passages of the seven conducted. The incubation of the cytopathogenic effect amounted to 7-9 days and was not lowered by the degree of subinoculation. The titers of the cytopathogenicity were low - an lg $TTsPD_{50}$ of 2.0-2.5, whereas the titers of the same cultural fluid in the mice reached an lg LD_{50} of 5.7. The virus's cytopathogenic effect was suppressed in the presence of the specific immune serum.

Evaluation of Results

In the cultivation of the tissues *in vitro*, all of the tissues selected for the test proved to be susceptible to the tick-borne encephalitis virus. The virus adapted readily to all of the tissues tested and reproduced from the first passage. Viral propagation was observed in the cultures of tissues from animals that are highly and slightly susceptible to tick-borne encephalitis. White-rat and dog tissues were used as the tissues of animals that are weakly susceptible to tick-borne encephalitis. In the cultures of skin-muscle tissue of a white-rat embryo the viral titers reached $10^{5.7}$ and the virus displayed a cytopathogenic effect. The cultures of skin-muscle tissue of mice, in spite of the high susceptibility of mice to the tick-borne encephalitis virus *in vivo*, proved to be a less suitable medium for the virus's propagation. The maximum viral titers achieved on this tissue were 10^4. As is well known, the first stage in the virus's propagation cycle is adsorption of the virus by the cell. Probably, one of the factors of cellular susceptibility to the virus is the different ability of the cells to adsorb the virus, whereupon this ability evidently differs in the same tissues *in vivo* and *in vitro*.

A comparatively wide spectrum of epithelian and connective tissues from the various animals proved to be suitable for the propagation of the tick-borne encephalitis virus. The cytopathogenic effect, however, was noted only in the cultures of fibroblasts from the skin-muscle and kidney tissues of the human embryo and the white rat. The virus acquired a high selectivity in the development of the cytopathogenic effect on the tissues *in vitro*. It was not registered in the cultures of human lung tissue in spite of a preeminent growth of fibroblasts. Evidently, the fibroblasts of the lung and skin-muscle tissues, while having a single morphological structure, differ in their metabolism *in vitro*, and lack the necessary conditions for the development of the mechanism stipulating the virus's cytopathogenic effect.

Of the tissues on which the virus did not show a cytopathogenic effect, a strain of HeLa cells proved to be the most sensitive to the tick-borne encephalitis virus. The viral titer in the cultural fluid on the HeLa cells was just as high (lg LD_{50} of 6.5) as in the cultures of human skin-muscle tissue, on which a cytopathogenic effect was developed. The absence of the virus's cytopathogenic effect on the HeLa cells is explained, possibly, by the epithelian origin of this strain. It is not clear whether the HeLa cells' high sensitivity to the virus is related to their origin from a malignant tumor.

We did not propose a comparative study of cultivation of the virus on embryonic and postnatal tissues of any sort of animal. Basically, we used embryonic tissues. The monkey and dog kidneys and the HeLa cells were postnatal tissues. There was no relationship observed between the reproduction of the virus and the age of the tissue. The viral titers in the culture of monkey kidney tissue and in the culture of the chick embryo were about the same.

Thus, the cytopathogenic effect of the tick-borne encephalitis virus was registered only in the connective-tissue elements of the human embryo and the white rat. A cytopathogenic effect was not noted in a single tissue of epithelian origin. The lung tissue of the human embryo, the fibroblasts of white mice, which are highly sensitive in vivo to tick-borne encephalitis, and the fibroblasts of the chick embryo proved to be unresponsive for the development of the virus's cytopathogenic effect. The ability of the tissue to produce the cytopathogenic dffect was unrelated to the degree of the animal's susceptibility to tick-borne encephalitis in vivo. Also, the intensity of the viral propagation did not show an effect on the cellular destruction. The HeLa strain, on which the virus reached high titers, preserved morphological stability during the cultivation of the virus.

Conclusions

1. The tick-borne encephalitis virus is successfully reproduced in tissue cultures of the human embryo, HeLa cells, monkey and dog kidney tissue, skin-muscle tissue of white mice and rats, and in cultures of the various tissues of the chick embryo.

2. The maximum viral titers in all of the tissue cultures are observed on the 3rd-8th day after inoculation.

3. The most intensive propagation of the virus is noted in the cultures of the human skin-muscle tissue and the HeLa cells.

4. A cytopathogenic effect is registered regularly in the cultures of human skin-muscle and kidney tissues on the 2nd-4th day after inoculation.

5. The viral titers determinable according to the cytopathogenic effect coincide with the titers on the mice.

6. The virus's cytopathogenic does not occur in the presence of serum from a tick-borne encephalitis patient.

7. A cytopathogenic effect is registered in some passages in cultures of the embryonic skin-muscle tissue of the white rat.

8. The virus's cytopathogenic effect is not developed in cultures of human lung tissue, HeLa cells, monkey and dog kidney tissue, skin-muscle tissue of white mice, and tissues of the chick embryo.

Footnotes

1/ According to our observations an active virus was preserved in the cultures of this type for a period of 35-44 days.

2/ The cumulative titer represents the sum of the viral titers in the cultural fluids of 40 consecutive passages.

3/ $TTsPD_{50}$ is the dose producing a cytopathogenic effect in 50 % of the test tubes containing the tissue cultures (the titer of the virus in the testing in the cultures of tissue).

4/ The calculation of the intensity of cellular degeneration was made by the plus system.

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Table 1

The pattern of the accumulation of the tick-borne encephalitis virus in cultures of human and animal tissues (lg LD_{50} according to Reed and Meusch).

Tissue	Day after inoculation							
	2nd	3rd	4th	5th	6th	8th	10th	12th
Human embryonic skin-muscle	4.5	6.3	6.3	6.0	6.0	5.7	4.0	4.0
Human embryonic kidney	4.5	4.5	4.5	4.0	3.7	3.5	-**	-
Human embryonic lung	4.7	-	5.5	-	5.5	5.5	-	-
HeLa cells	6.5	6.5	6.5	6.0	5.5	5.0	4.5	-
Monkey kidney*	4.0	6.0	-	5.0	4.5	4.0	4.0	-
Dog kidney*	4.0	5.0	5.0	5.0	4.0	3.0	-	-
White mouse embryonic skin-muscle	4.0	-	3.3	-	3.0	2.5	-	-
White rat embryonic skin-muscle	4.5	-	4.7	-	5.5	4.5	5.7	-
Chick embryonic skin-muscle	-	5.7	5.0	5.0	4.0	3.0	3.0	-
Chick embryonic heart	-	6.0	5.5	5.5	5.0	4.0	-	-
Chick embryonic lung	2.5	3.0	3.0	-	4.0	3.0	-	-
Chick embryonic chorioallantoic	4.5	6.0	6.0	6.0	5.0	5.0	-	-

* Material from the 4th passage

** Investigation not conducted

Table 2

The data of the comparative titration of the tick-borne encephalitis virus in cultures of human embryonic skin-muscle tissue and on mice.

Titration	Day after inoculation						
	2nd	3rd	4th	5th	6th	8th	10th
In the tissue cultures in lg $TTsPD_{50}$. . .	5.0	7.5	7.0	6.5	6.3	5.0	3.0
On mice in lg LD_{50}. . .	4.0	7.5	6.7	6.5	6.0	5.0	4.0

Table 3

The neutralization test for the tick-borne encephalitis virus in a culture of human embryonic skin-muscle tissue with the serum of Patient D.

Serum			Dilution of the serum								Controls			
Day of disease	Neutralization indices on mice	Antigen for the neutralization test	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	No. 1	No. 2	No. 3	Titer of Serum
5th	316	1000 $TTsPD_{50}$ (brain antigen)	----	----	++--	++++	++++	++++	++++	++++	++++	++++	++++	1:16
		1000 $TTsPD_{50}$ (cultural antigen)	----	----	+---	++--	++++	+++	+++	+++	+++	+++		1:16
15th	4677	1000 $TTsPD_{50}$ (brain antigen)	----	----	----	----	----	----	+++-	++++				1:256
		1000 $TTsPD_{50}$ (cultural antigen)	----	----	----	----	----	----	+++-	++++				1:256

Denotation: + presence of a cytopathogenic effect in the test tube;
- absence of a cytopathogenic effect in the test tube.