

AD733638

TRANSLATION NO. 2781

DATE: 2 December 1971

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The factors determining the level of virus production.

Report 1: The influence of infection multiplicity on virus production in the case of VEE (Virus of equine Venezuelan encephalitis)

A.S. Novokhatsky, V.I. Ershov, D.I. Ivanov Institute of Virology, Academy of Medical Sciences, Moscow/ Pub. April 3, 1969

This is a study of the influence of the conditions of cultivation and infection multiplicity on the level of production of the virus of equine Venezuelan encephalitis (VEE) using trypsinized fibroblasts of chicken embryos. Use was made of monolayer stationary, roller, and suspension cultures of previously trypsinized cells. The biggest harvest was received in roller cultures. A decrease in infection multiplicity regularly led to growth of the final titers of the VEE virus. We discuss the mechanism determining the revealed phenomenon, including the possible role of the inoculated and newly formed interferon in the process of virus replication.

The size of the average output of virus for the first infected cell varies from one unit (parotitis virus, tick encephalitis etc.) to a thousand (adenoviruses, the smallpox virus, etc) (10). The so-called virus harvest is determined by the complex of peculiarities of virus reproduction, the size of the virions, and their chemical composition.

However, besides the existing distinctions in the character of formation and liberation of the virions, there is, for the representatives of various taxonomic groups, an expressed variability in the size of the output for one or another type of virus. This size depends on the multiplicity of concrete factors: growth and sensitivity of the cells used in the experiment, the incubation temperature and time, the composition of the feeding medium, the method of cultivation, and others.

It is important to emphasize that research done on the degree of influence of each separate factor on the production of a virus has conditional value in application, since development of optimal conditions for receipt of viruses with maximal indicators of biological activity is of course, the goal of producing virus preparations (vaccine, diagnostic agents, and others).

We studied the influence of conditions of cultivation and infection multiplicity on the level of virus production. In strictly defined conditions which were optimal for each method of cultivation, and which were described in our other works (3, 5, 8), we changed one of the parameters under studied. As a basic test, we used determined magnitudes of virus output, arising from the fact that this test is a good summary indicator of the validity of all the foregoing stages of the ontological development of a chosen model virus.

Materials and Methods

We used the virus of equine Venezuelan encephalitis (VEE) received from the Rockefeller center in 1944 and supported since then in the Ivanov Institute of Virology by serial passages in mice and chicken embryo fibroblasts. The virus was titrated using the method of platelets under agar (11).

We used previously trypsinized chicken embryo fibroblasts (FKE cells) which were prepared in the usual way as described by O.G. Andzhaparidze and his co-authors (1).

For receipt of monolayer stationary cultures, cells were diluted in a medium consisting of medium No. 199 and a 5% solution of hydrolyzate lactalbumin (1:1) and containing 5 - 7% of normally warmed bovine serum and the antibiotics penicillin and streptomycin at 100 units/milliliter (growth medium), and up to a concentration of $1 \cdot 10^6$ cells/ml. The suspension of cells was poured into 100 gram flasks, 10 ml. in each one. After formation of the monolayer (usually in 48 hours), the growth medium was poured off, and the cells washed twice with a Henkes solution and the corresponding virus dilution in Henkes solution introduced into matras flasks in 2 ml. quantities. The contact of the virus with the cells at 37 degrees continued for 30 minutes. Then the virus was drawn off, the cells washed in Henkes solution and a medium of aggregation poured on (medium no. 199 with 10% bovine serum and antibiotics). In 24 hours, the cultivation liquid was poured off and then we determined the infection titer of the virus contained in it.

For roller cultures we used 1 liter bottles of jena glass, the revolution of which was insured by special stands which were two layered bases with rollers turning around a horizontal axis (9). The suspension of cells in the growth medium with a concentration of 3 mln. in 1 ml. was introduced from a calculation of 1/10 of the volume of the bottle. The formation of the monolayer took place as a rule in about 48 hours, after which the bottles were taken from the stand, the growth medium poured off, and the cells washed twice with Henkes solution. The corresponding solution of viruses in the Henkes solution was introduced in the volume of 10 ml. After contact for 40 minutes at 37 degrees and a constant revolution, the virus was drawn off, the cells washed and introduced into each bottle of aggregation medium at a calculated 1/20 of the volume of the bottle. In 24 hours afterwards, the culture liquid was poured off and the virus which it contained was determined.

In cultivation of suspension cultures, the cells were resuspended in 20 ml. of Henkes solution, containing a corresponding quantity of virus. The contact took place at 37 degrees and constant mixing for 40 minutes. Then the cells were centrifuged at 2000g for 15 minutes. The settling liquid was poured off and the cells washed three times with Henkes solution to remove unadsorbed viruses. The precipitation of cells was suspended again in growth medium, diluting them to a density of $3 \cdot 10^5$ cells per ml. The cultivation was done in flasks with mixing of the suspension with a magnet suspended on a chain (spinner system), or on a stand for bathymetric cultivation (7). The samples were taken away in 24 hours and centrifuged for 20 minutes at 6000 g and the determination made of the virus content in the settling liquid.

A calculation of the infection multiplicity is very simple in the case of a suspension culture, and represents a specific difficulty in virus replication in roller and stationary cultures. We started with the supposition that for monolayer cultures of previously trypsinized cells the index of proliferation is near 1, as was shown in a series of experimental works (5, 2), therefore the multiplicity of infection in this case was determined by the seeding dose. This basis served for a calculation of the quantity of cells in 1 ml. of culture medium. In suspension cultures the density of the cell population was determined by calculation in a

Goryaev compartment.

The activity of the interferon was determined by degrees of pressure of platelet formation in connection with the VEE virus (4).

Results

Influence of infection multiplicity on the production of the VEE virus in various conditions of cultivation. Monolayer stationary, roller and suspension cultures of FKE cells were infected and inoculated with a corresponding quantity of subsequent 10-multiple solutions of VEE virus in Henkes solution in order to create an infection multiplicity gradient. The titer of the virus in an aggregation medium was determined after 24 hours of incubation, since in this time the virus titers in the presence of infection by a defined dilution already reached a maximum or approached it, and the subsequent decrease of thermoinactivation of titers during infection with massive doses was still insignificant. In sketches 1 - 3, is represented the curve of change in the virus titers depending on the infection multiplicity in various culture systems. Each point on the graph is the average arithmetic result of three parallel experiments, with the exception of sketch 3 where each experiment is shown.

In cultures of previously trypsinized cells the decrease of infection multiplicity regularly leads to the growth of maximal titers. This effect is best expressed in roller cultures where the difference in virus production during infection by a massive dose and limited dilution reaches 1,5 - 3 lg. Less noticeable is the influence of infection multiplicity in suspension cultures which in general give a rather large range of results. The most fuzzy differences are observed in monolayer stationary cultures although even here there is a clear tendency towards increase in the virus output during decrease of the infectious dose. Slowed growth of maximal titers in roller and suspension cultures during infection multiplicity is $1 - 1 \cdot 10^{-3}$ PFU/cell and so expressed in stationary monolayer, that the curve takes on a two humped appearance. For monolayer stationary cultures, a decrease of the harvest during infection with limited dilutions of the virus is also characteristic.

For clarification of the nature of the processes leading to the formation of the observed types of curves of the growth of the VEE virus in cells, we introduced a special series of experiments. Monolayer stationary cultures of FKE cells were infected with a growth dilution of virus and in 24 hours we defined the level of growth of the interferon. The results presented in sketch 4 show that the maximum of the interferon is produced during infection multiplicity of $1 - 0,01$ PFU/cell. This closely corresponds with the first decrease in the harvest curve of the virus and suggests that one consider the influence again of the interferon being produced conditional to this decrease. In contradiction to this, it should be noted that in roller cultures, the titers of the interferon can reach a very significant magnitude even during infection with limited doses of the virus (see table).

Discussion

Inasmuch as Hardy and Brown noted that during infection^(of) the L-cell with the VEE virus no autointerference was observed even during infection multiplicity at 400 PFU/cell, a comparatively low value of harvest during infection with massive doses of virus can be explained by the influence of the interferon introduced together with the virus. Our experiments on the determination of the concentration of the interferon in the series

of model viruses (VEE) used showed that during infection with the infection multiplicity at 1 - 10 PFU/cell, the culture undergoes activity of the interferon at a quantity of 1 - 2.5 immunization units⁵⁰. Moreover, in specially conducted experiments it was established that during infection of monolayer cultures of FKE cells with a virus, which does not contain homologous interferon (we used a virus passivated in L-cells), when in this way its primary activity is excluded, the dependence of virus production on infection multiplicity takes on a completely different character. In this case there is no note of any decrease in the output of virus during infection with massive doses, and the maximal titer is 2 - 4 times higher than the control figure received in cultures infected with a high infection multiplicity by viruses containing homologous interferon.

Infection multiplicity is one of the many factors influencing the magnitude of virus output. The increase of the maximal titers of the virus during a small infection multiplicity is noted for various viruses (6). However the literature statistics on this question are only piecemeal and at times contradictory.

We suppose that the influence of infection multiplicity on the final output of the virus can be to a known degree conditional to the participation of the interferon. Really, the titers of the forming interferon are directly proportional to the infection multiplicity of the cells with the virus VEE (4). During infection of the FKE cells with the Semlik forest virus, the maximum of production of the interferon reaches an infection multiplicity of 0.2 PFU/cell, for which the production curve of the interferon in dependence on the infection multiplicity has to a limited degree an expressed peak.

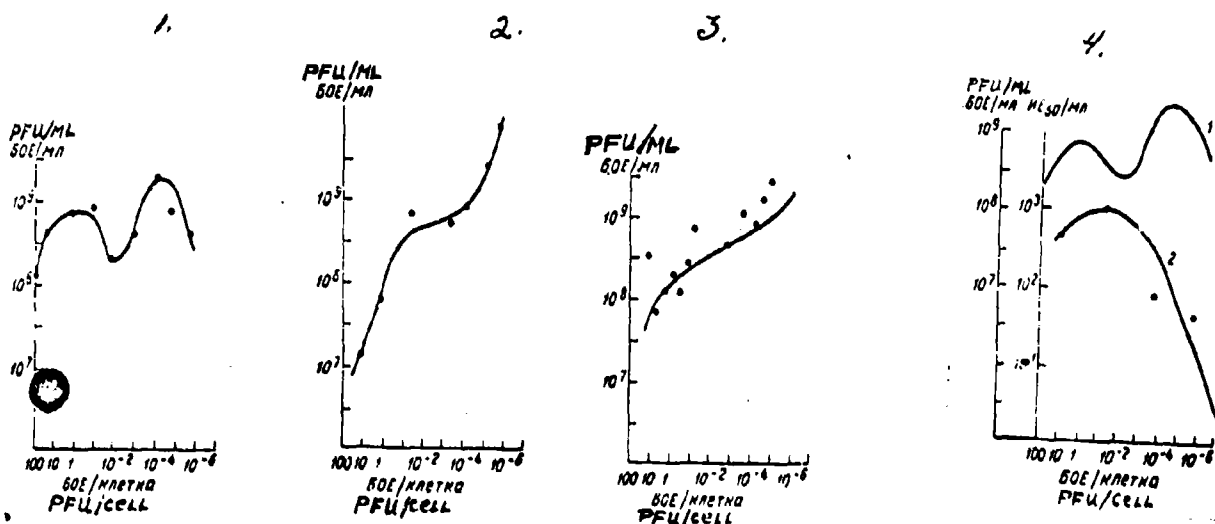
In analyzing the results, it is necessary to differentiate clearly the action of the introduced and the reformed interferon. If the influence of the introduced interferon, naturally, is revealed on any type of cultivation, the again formed interferon acts chiefly on the monolayer stationary method of cultivation.

Besides the inhibiting action of the interferon, the increase of the maximal titers of the VEE virus during the use of limited small infectious doses and the variability of this phenomenon depending on the method of cultivation can explain a series of circumstances: 1) inasmuch as in this case there have been observed many cycled virus replications, an indispensable condition for the appearance of this phenomenon is a sufficiently short cycle of virus reproduction and a high sensitivity of the cells which has been noted during the use of the chosen method; 2) during a small infection multiplicity, the again forming virus to a lesser degree undergoes thermoinactivation, since the peak of infection in connection with the many-cycled nature of the process is observed in the later hours; 3) mixing of the feeding medium in roller and suspension cultures eases the infection of the cells; 4) infection with small doses in the case of suspension cultures of previously trypsinized cells allows these last to adapt somewhat to the conditions of existence in a suspension; 5) the expressed cytopathological effect, quickly leading to the total destruction of infected cells which is usually observed during infection with massive doses, especially for roller and suspension cultivation, also reacts negatively on the magnitude of the virus harvest; on the other hand, in cultures infected with small doses of virus, the cells die significantly later and they produce a larger quantity of virions.

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- Sketch 1. Accumulation of VEE virus in monolayer stationary cultures depending on infection multiplicity.
- Sketch 2. Accumulation of the VEE virus in roller cultures depending on infection multiplicity.
- Sketch 3. Accumulation of VEE virus in a suspension of FKE cells depending on infection multiplicity.
- Sketch 4. Production of VEE virus (1) and interferon (2) in FKE cells depending on infection multiplicity.



Production of the interferon in roller cultures of chick embryo fibroblast cells.

Infection multiplicity (in PFU/cell)	Virus titer (in lg PFU/ml)	1 hemagglutinin titer	Interferon titer (in IU ₅₀ /ml)
1 - 10	8,7	1 024	640
	8,3	512	1 280
1·10 ⁻⁵ - 1·10 ⁻⁶	9,0	256	640
	9,5	1 024	640
	9,1	512	1 280
	9,7	2 048	1 280