THE MATURATION OF WESTERN EQUINE ENCEPHALOMYELITIS VIRUS AND ITS RELEASE FROM CHICK EMBRYO CELLS IN SUSPENSION

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A method for assaying animal viruses by producing necrotic plaques on a monolayer of cells *in vitro* was developed recently, and applied to a detailed study of the growth characteristics of Western equine encephalomyelitis (W.E.E.) virus in cell suspensions (1). The measurements in that study were confined to extracellular progeny virus. As measured in those experiments, no new virus could be detected in the medium for about 3 hours after infection. Then an exponential increase began, and continued for 2 to 3 more hours, after which no additional increase was detected. Experiments with single infected cells indicated that virus was released gradually over a period of several hours.

The present report deals with the maturation of intracellular progeny virus and the rate of its release from the cell.

Materials and Methods

Virus.—A strain of chick embryo–adapted Western equine encephalomyelitis (W.E.E.) virus was used in all the experimental work, which was the same strain used by Dulbecco and Vogt (1). Virus stocks were prepared by infecting a monolayer tissue culture of chick embryo cells (1) and collecting the supernatant 14 hours later. This stock was kept frozen at −10°C. until ready for use, and had a titer of about 2×10^9 plaque-forming units per ml.

Virus Assay.—Virus was assayed by the plaque technique (1). Monolayers of chick embryo cells were prepared according to the following procedure. 10-day-old chick embryos were disrupted by forcing them through a wire mesh screen at the bottom of a 50 ml. syringe. The resulting pulp was digested with 0.25 per cent trypsin in Earle's saline (E.S.[1]) (1); the resulting cell suspension was washed twice by centrifugation and resuspension in E.S. About 10^7 cells were placed on a Petri dish. 2 ml. of horse serum and 1 ml. of chick embryo extract were first added followed by E.S. to make a final volume of 10 cc. The cells attached to the glass surface, and after 48 hours constituted a continuous cell layer.

To assay virus samples, the medium was removed and the cells washed twice with 5 ml. of phosphate buffered saline (P.B.S.[2]) (1). Then, 0.6 ml. of a virus dilution in P.B.S. cal-

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[1] Earle's saline.

[2] Phosphate buffered saline.

culated to give between 50 to 200 plaques was spread over the cells; the plate was then incubated for 30 minutes to permit adsorption of the virus onto the cells. After adsorption the cell layer was covered with 3.7 ml. of an agar overlay kept at 44°C., and containing 0.9 per cent washed agar in a mixture made up of 14 per cent chick embryo extract in E.S. The overlaid plates were incubated for 2 days at 37°C. in an atmosphere of 4 per cent CO_2 in air. To detect the plaques, 5 ml. of 1:10,000 neutral red in E.S. was added over the agar at the end of the incubation period. After a 2 hour period of further incubation at 37°C. to permit diffusion of the stain through the agar and uptake by the cells, the plaques were counted. They stood out as round, colorless areas of necrotic cells on a dark background of stained, living cells.

Assay of Infected Cells.—To determine the proportion of cells which were infected and able to release virus, the cells were assayed during the latent period, that is to say before each cell had released an average of one virus particle. The technique was the same as that employed in virus assay. Under these conditions each infected cell gave rise to a single plaque, owing to virus which the cell liberated after it was placed on the cell layer.

Cell Suspensions.—The method for preparing cell suspensions from the chick embryo monolayers is described in the Experimental section.

Disruption of Cells.—To assay intracellular virus, washed cells were disrupted by ultrasonic vibration in the constant temperature water bath of a hypersonic generator[3] which employed a saucer-shaped barium titanate crystal to focus the wave beam. The frequency was 600 kilocycles per second, total power 200 watts, primary voltage 125, R.F. current 6.0, and oscillation setting at 0.1. Rubber-stoppered pyrex tubes were immersed in the water bath at the point of maximum wave energy as determined by the size of the fountain produced in the contents of the tube.

EXPERIMENTAL

Efficiency of Ultrasonic Vibration in Disintegrating Cells.—The effect of ultrasonic vibration on chick embryo cells was determined by direct observation and counting of the cells in a hemocytometer before and after treatment. A 1 ml. suspension containing 10^7 normal chick embryo cells in P.B.S. was subjected to ultrasonic vibration for 2 minutes. More than 99.9 per cent of the cells were disintegrated into very small fragments by this treatment, thus proving it to be highly efficient in breaking open the cells.

Effect of Ultrasonic Vibration on Virus Infectivity.—To determine whether ultrasonic vibration influences the infectivity of the virus, 0.1 ml. of the stock virus was diluted in 0.9 ml. P.B.S. and exposed to ultrasonic vibration for 2 minutes. It was then assayed, and its titer compared with that of a control virus sample which had not been vibrated. To test any indirect effects that the products of cell disruption may have had on the virus infectivity, 6×10^6 cells in 1 ml. of P.B.S. were vibrated and mixed with 0.1 ml. of virus suspension. The mixture was kept at 25°C. for 5 minutes and then in an ice bath until inoculated, to simulate the conditions under which virus would be exposed to such fragments in later experiments.

The results of these experiments are presented in Table I. There was no evidence of either direct or indirect effect of ultrasonic vibration on virus infectivity.

[3] Hypersonic transducer model 301 manufactured by the Brush Development Company, Cleveland.

Intracellular Appearance and Release of W.E.E. Virus:

A 2 day old monolayer culture of chick embryo cells was washed twice with P.B.S. and all the fluid removed. 0.1 ml. of stock virus, containing about 2×10^8 plaque-forming particles were diluted in 0.5 ml. of P.B.S. and the entire suspension added to the culture. After 30 minutes at 37°C. for adsorption, the monolayer was washed four times with 10 cc. of P.B.S. to remove unadsorbed virus. This was followed by the addition of 10 cc. of 0.025 per cent trypsin in E.S. which detached the cells from the glass. The suspension was then pipetted up and down to break up cell clumps and obtain a uniform suspension of isolated cells. These suspended cells were washed three times by centrifugation at 1000 R.P.M. for 2 minutes in an International size 1 type SB centrifuge, and resuspension in 10 ml. of E.S. to remove the trypsin and any remaining unadsorbed virus. As a consequence of the repeated washing of the cells after infection, both on the monolayer and in suspension, the infecting virus was diluted by a factor greater than 10^{10}.

TABLE I

Direct and Indirect Effects of Ultrasonic Vibration on W.E.E. Virus Infectivity

Material	Dilution factor	No. of plaques	Titer
Untreated virus	10^7	196,256	2.26×10^9
Virus exposed to ultrasonic vibration	10^7	192,216	2.04×10^9
Virus incubated with cells exposed to ultrasonic vibration	1.1×10^7	196,177	2.06×10^9

The cells were counted in a hemocytometer and diluted to a final concentration of 5×10^4 cells in 50 ml. of a solution containing 20 per cent chick embryo extract in E.S. Thus double protection was provided against reinfection. The cells were infected with a high virus multiplicity to insure infection of all the cells, and the cells were diluted to such an extent that the opportunity for reinfection became negligible.

The cell suspension was then placed in a 37°C. incubator flushed with a 4 per cent CO_2 in air mixture.

The moment of dilution was considered as time zero, although the moment at which virus had been added occurred approximately 50 minutes earlier. This was done because it was previously shown that W.E.E. virus does not reproduce in diluted cell suspensions unless some external growth factor such as embryo extract is added (1).

It is possible that some virus development occurred before zero time under the conditions of the present experiment, since the cells remained in high concentration on the monolayer during the adsorption period, and Pereira has reported that the virus of fowl plaque may reproduce in a concentrated cell suspension in the absence of external growth factors (2). The uncertainty about the precise moment when virus development is initiated does not influence the significance of the experiments, now to be described, since they were chiefly concerned with a comparison of the amount of virus present outside of and within cells in the same culture.

3 ml. samples were removed from the suspension of infected cells at hourly intervals and treated as follows:—

1. 0.1 ml. was diluted directly and plated to measure the total number of infected cells plus newly released plaque-forming virus particles.

2. The remaining 2.9 ml. were centrifuged at 1000 R.P.M. for 2 minutes and the supernatant was removed and assayed to determine the quantity of released virus alone.

3. The sedimented cells were washed again by centrifugation in 3.0 ml. of P.B.S. to further remove released virus and finally resuspended in 1.5 ml. of P.B.S. They were exposed to ultrasonic vibration to release intracellular virus, which was then assayed.

The curves resulting from the three separate assays over an 8 hour period in a typical experiment are plotted in Fig. 1.

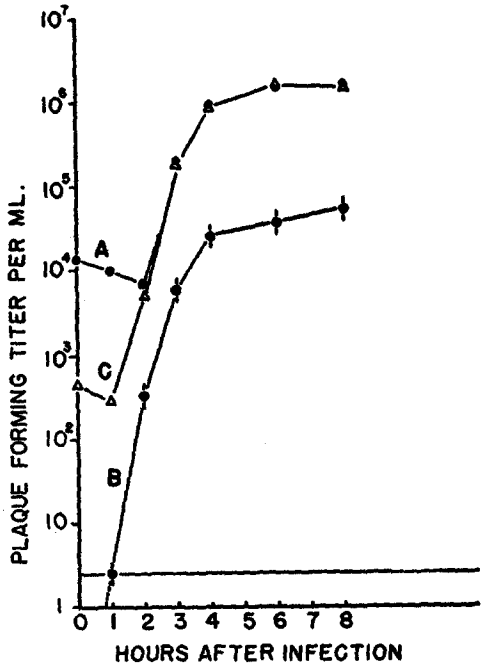

FIG. 1. Intracellular appearance and release of W.E.E. virus from chick embryo cells A, intact cells plus released virus; B, intracellular virus (virus artificially released from washed and sonically vibrated cells); C, extracellular virus (virus naturally released into the medium).

As shown in curve B, Fig. 1, no infective virus could be found associated with the cells at time zero in spite of the fact that there were 1.3×10^4 infected cells per ml., as indicated in the plot of the unwashed cell suspension A. Both intracellular and supernatant infective virus started to increase exponentially between the 1st and 2nd hour, which was about 1 hour before any rise could be detected in the unwashed cell suspension. This discrepancy was due to the large background of infected cells in the unwashed cell suspension, since the number of newly released infective virus particles could be measured only after it be-

came comparable to the number of infected cells. However, the newly released virus could be measured if the background of infected cells was eliminated either by centrifugation or destruction of the cells by ultrasonic vibration, as was done in the case of curves B and C.

The data in Fig. 1 were used to measure the average time which elapsed between the moment a virus particle acquires the property of infectiousness within a cell and the moment it is released from that cell into the surrounding medium, called here the "release time." Particular attention in this consideration was directed to the period of exponential increase of both intracellular and extracellular virus. By doing so, the importance of thermal inactivation of the released virus was limited, since the exponential period is short, and the amount of virus inactivated insignificant. Furthermore, this procedure permits a convenient calculation of the release time as shown below.

The basic assumption on which the calculation is based is that extracellular infectious virus is produced as a consequence of two successive steps: intracellular maturation of virus, followed by release into the medium. Under this assumption the rate in increase of extracellular virus $\frac{dc}{dt}$ is at any instant proportional to the concentration of the intracellular virus $B(t)$ present at that instant, and to a velocity constant K_1; $\frac{dc}{dt} = K_1 B(t)$.

K_1 represents the probability per unit time that an intracellular virus particle will be released. Therefore, its reciprocal, $1/K_1$, is the average time required for a virus particle to be released once it has become infective, or the "release time." Both $\frac{dc}{dt}$ and $B(t)$ can be determined from the experimental curves given in Fig. 1. Furthermore, it is shown in Fig. 1 that intracellular virus, B, and released virus, C, increase at the same rate, and that B remains a constant fraction of C during the period of exponential virus release. The latter finding allows the substitution of $\frac{C}{r}$ for B.

Thus $\frac{dc}{dt} = \frac{K_1}{r} C$. The solution of this differential equation is $1nC_2 - 1nC_1 = \frac{K_1}{r}(t_2 - t_1)$ and

$$1/K_1 = \frac{t_2 - t_1}{r(ln\ C_2 - ln\ C_1)}$$

in which

$1/K_1$ = average "release time."

$r = \frac{C}{B}$ the constant ratio of extracellular virus C to intracellular virus B, at any time during the period of exponential virus release.

t_1, t_2 = two arbitrary times during the period of exponential virus release.

ln C_1,lnC_2 = natural logarithm of concentrations of extracellular virus at times t_1 and t_2, respectively.

The calculated "release time" varied from 0.4 to 2 minutes, averaging 0.9 minutes over four separate experiments, indicating that virus is released very rapidly under these conditions, after it gains the property of infectiousness.

An approximate value of the release time can be obtained graphically. Thus, the quantity of virus found within the cells, B, for example at 2 hours, (3×10^2), can be added graphically to the released virus, C, present at that moment (4.8×10^3), by constructing a vertical line to 5.1×10^3. A horizontal line can then be extended to the right from this point to meet C. The time difference between this point of intersection and the 2 hour point is the time elapsed until all the virus present in the cells at 2 hours is released and appears in the supernatant, that is to say, the "release time." Graphically determined, the approximate release time arrived at in Fig. 1 is less than 1 minute.

It is of interest to note that the average maximum number of virus particles associated with each cell during the period of exponential increase was very small varying from 4 to 10 particles in the four experiments, in spite of the fact that each cell had spontaneously released an average of more than 100 virus particles by the end of this period.

DISCUSSION

Ultrasonic vibration produces a fragmentation of suspended cells into very fine debris without reducing virus infectivity and hence should be an efficient method for freeing virus particles which have reached full development, but have not yet been released from the cell.

The disruption of cells shortly after infection with W.E.E. virus in the work reported here showed that the infecting particles lost their infectiousness soon after their adsorption on the cells; no intracellular infectious particles could be detected in more than 10^4 infected cells fragmented during the first hour after adsorption. A similar loss of infectiousness following adsorption has been described for influenza virus adsorbed on the allantoic membrane of the chick embryo (3). There, however, the washing away of unadsorbed virus was relatively inefficient, and some residual infectious virus always remained in the tissue. Furthermore, the number of infected cells had to be determined indirectly. These complications were overcome in the present experiments: the suspended cells could be thoroughly washed, and the number of infected cells determined directly.

Between the 1st and 2nd hour an exponential increase started in both intracellular and released virus, and continued at about the same rate for 3 hours, the virus doubling in amount every 15 minutes. The first part of this rise could not be detected when both intact cells and released virus were assayed together by the plaque technique because of the large background of infected cells capable of producing one plaque each. Accordingly the first progeny virus appears in this system about an hour before the end of the latent period as determined in Dulbecco and Vogt's experiments (1). Still, it seems appropriate to consider Dulbecco and Vogt's figure as the true latent period, since it is the time when each cell has, on the average, produced one virus particle.

The ultrasonic disintegration of the infected cells washed free of extracellular

virus, released only small quantities of virus; the maximum number of particles released was 4 to 10 per infected cell at the end of the period of exponential multiplication, by which time every cell had already spontaneously released an average of more than 100 particles. These facts taken together suggest that the rate of release is rapid. On direct calculation from the experimental data to determine the "release time" (the average time elapsed between the completion of an infectious particle within the cell and its release from the cell), it was found to be shorter than 1 minute when results from four experiments were averaged. Data suggesting that influenza virus hemagglutinin is also released from the cells of the allantoic membrane of the chick embryo very shortly after its maturation have been reported by Cairns and Mason (4). These findings when taken together are consistent with the interpretation that the final stages of development of the virus of W.E.E. and of influenza occur near the periphery of the cell. However, they do not rule out the possibility that virus maturation is completed elsewhere in the cell, with the particles rapidly reaching the periphery by streaming or diffusion.

The "release time" may be much longer in other animal virus systems, such as the pox viruses, herpes, and psittacosis, instances in which there is ample cytological evidence for large numbers of intracellular particles, morphologically indistinguishable from the infective virus.

SUMMARY

Experiments are presented in which the plaque assay technique was used to study the intracellular appearance and release of Western equine encephalomyelitis virus in suspensions of infected chick embryo fibroblasts.

No intracellular virus could be found during the 1st hour after adsorption in spite of the fact that more than 10^4 cells per ml. proved to be infected. This is taken to indicate that the infecting particle loses its infectivity upon entering a susceptible cell.

The first progeny virus was detectable in the cells between 1 and 2 hours after infection, and it increased in amount exponentially during the following 3 hours. The released virus as measured in the supernatant fluid increased at the same rate as the intracellular virus but exceeded it in amount by a factor of about twenty at all times during the period of exponential increase. More than 100 particles were spontaneously released from each cell, by the end of the period of exponential increase, yet the maximum number of intracellular infective particles at any instant during this period was never more than an average of from 4 to 10 per cell. Calculations based on these findings indicate that, on the average, a virus particle is released from the cell within 1 minute after it gains the property of infectiousness.

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