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STUDY OF PLAQUE TITRATION OF VACCINIA VIRUS IN CHICK EMBRYO CELL CULTURES. II. ASSAYS BY INOCULATION OF A CELL SUSPENSION AND IN LIQUID MEDIUM

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Summary. — Inoculation of a chick embryo cell suspension allows effective adsorption of infectious vaccinia virus units, unlike inoculation of a preformed cell monolayer, in which adsorption is slow and incomplete. The curve of the increase in the number of plaques in cells maintained under liquid medium is continuous. The rate of plaque number increase is inversely correlated to the number of cells in the culture; it is influenced by the incubation temperature, the optimal temperature being 37° C. Andersen and Larsen's technique can be recommended as the most convenient method of plaque titration of vaccinia virus in a chick embryo cell culture. The results obtained by this technique are also influenced by the number of cells in the culture and by the incubation temperature. The $NaHCO_3$ concentration does not affect plaque formation within limits of 0.05 to 0.2%.

Introduction

In an earlier paper (Slonim and Hulenová, 1966) we showed that inoculation with vaccinia virus of a cell suspension, obtained by trypsinization of chick embryos, was a satisfactory method for determining the virus titre (TCD_{50}) in tube cultures. We also found that this titration method gave the same results as titration on chick chorioallantoic membranes (CAM). Andersen and Larsen (1966) used inoculation of chick embryo cell suspension for plaque titration of vaccinia virus.

In the present experiments we inoculated cells suspensions with vaccinia virus and studied a number of factors influencing the formation of plaques "in liquid medium" or under an agar layer in Petri dishes, in relation to the titres determined on chick embryo CAM.

Materials and Methods

Cell cultures. A suspension containing a given number of cells obtained by trypsinization of the bodies of 10—11-day chick embryos was pipetted in 4 ml amounts into plastic Petri dishes (NUNC, Denmark) 5 cm in diameter, into which 0.1 ml virus inoculum had been pipetted just beforehand. The cultures were incubated at 37° C for given lengths of time. Before reading the results, the medium was decanted and the cultures were stained with carbol fuchsin, washed with water and dried; typical vaccinia virus plaques were counted under a magnifying-glass.

The culture medium was composed as follows: medium 199 (as modified by Slonim *et al.*, 1960) — 100 ml; heated calf serum — 2 ml; 7.5% $NaHCO_3$ solution — 0.7 ml; penicillin — 100 units/ml; and streptomycin — 100 μg/ml.

The other methods used were described previously (Slonim and Hulenová, 1969). PFU and CAM-PFU mean plaque-forming units determined in cell cultures and pock-forming units determined on chick embryo CAM, respectively.

Results

Time of incubation

In the following experiments plaque formation was studies in cell suspensions (6×10^6 cells were seeded per Petri dish) inoculated with vaccinia virus. The virus suspension, containing 1.1×10^8 CAM-PFU/ml, was used in 10^{-6}, $10^{-5.48}$ and $10^{-5.3}$ dilutions. Between the 48th and 72nd hour of incubation 8 dish cultures were stained at 4-hour intervals in each experiment and the mean plaque count was determined.

The curves in Fig. 1 were constructed from the mean values. These values were converted to the PFU count per ml of the original virus suspension and correlated to the CAM-PFU count. It can be seen that $\log_{10}$ of the plaque count, both with high and low inoculum dilutions, rose approximately linearly with time (lower part of Fig. 1). The CAM-PFU count was reached in this system after 63 hours' incubation (upper part of Fig. 1). After 68 hours, small satellite plaques, clearly of a secondary nature, appeared. Similar small plaques were also seen before this interval, but as they did not show the typical satellite arrangement they could not be definitely identified as secondary plaques.

Number of cells

As shown previously (Slonim and Hulenová, 1969), the number of seeded cells influences the plaque count in titration under agar overlay. We therefore also studied the influence of this factor on titration results "in liquid medium".

The cells were simultaneously seeded into Petri dishes in amounts of 4, 6, 8 and 12 millions per dish and infected with 0.1 ml vaccinia virus suspension (titre 1.1×10^8 CAM-PFU/ml) diluted $10^{-5.48}$. On the 2nd and 3rd day of incubation the mean plaque counts were determined in at least 4 cultures in each group. The resultant values calculated from the means of three such experiments as the PFU count/ml, were also correlated to the CAM-PFU count/ml (Fig. 2).

It is clear that the number of cells seeded into the dish influenced the rate of plaque formation and hence the number of plaques which can be seen and counted on a given day of incubation. There was an inverse correlation, since the number of plaques fell with rising number of cells; $\log_{10}$ of the plaque count was roughly a linear function of the number of cells. If the results were read after 48 hours' incubation, an increase in the number of seeded cells in steps of one million corresponded to an average decrease of 0.14 $\log_{10}$ in the plaque count.

On prolonging the incubation time (72 hours), the rate of plaque formation rose; it was highest in the presence of the maximal number of cells (1.19 $\log_{10}$)

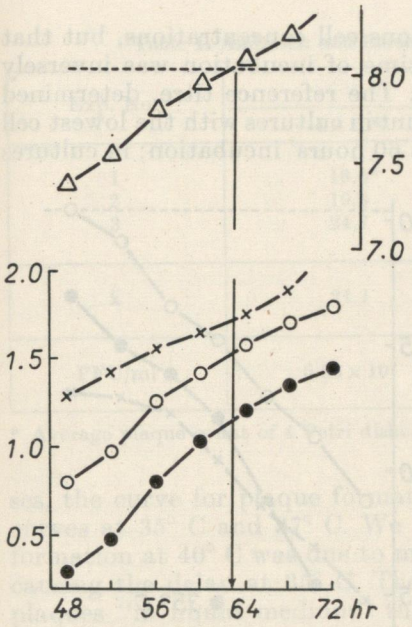

Fig. 1.

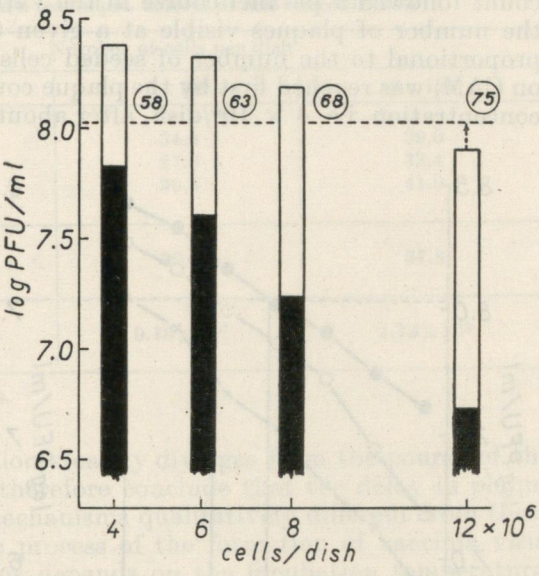

Fig. 2.

Fig. 1.

Time of incubation

Abscissa: incubation time in hours
Left ordinate: number of plaques per dish ($\log_{10}$); ●, ○, ×; three different virus input multiplicities
Right ordinate: $\log_{10}$ PFU/ml original virus suspension; △———△ average values of all experiments; ---------- $\log_{10}$ CAM-PFU/ml original virus suspension (reference)

Fig. 2.

Number of cells

Abscissa: number of cells seeded per dish
Ordinate: $\log_{10}$ PFU/ml original virus suspension
Black columns: $\log_{10}$ PFU/ml after 48 hours' incubation
White columns: $\log_{10}$ PFU/ml after 72 hours' incubation
---------- $\log_{10}$ CAM-PFU/ml orig. virus suspension (reference); figures in circles: time in hours at which the reference CAM-PFU value was reached

and fell progressively with decreasing cell concentration (0.96 $\log_{10}$, 0.75 $\log_{10}$, 0.56 $\log_{10}$). Satellite (secondary) plaques appeared in the reverse order, i.e. they were seen first in the presence of the minimal number of cells. Fig. 2 also gives the times at which it can be assumed that the plaque count will attain values corresponding to the titre (per ml) determined on chick CAM; these times rose together with the number of cells.

In further experiments we studied plaque formation in cultures containing 4, 6, and 8 million cells per dish and determined again the times at which each system attained the number of plaques corresponding to the CAM-PFU count. The results given in Fig. 3 show that the increase in $\log_{10}$ of the plaque

count followed a parallel course in the various cell concentrations, but that the number of plaques visible at a given time of incubation was inversely proportional to the number of seeded cells. The reference titre, determined on CAM, was reached first by the plaque count in cultures with the lowest cell concentration, i.e. 4×10^6/dish, after about 60 hours' incubation; in cultures

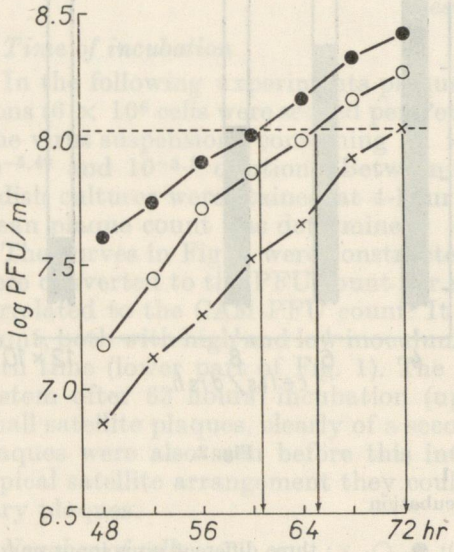

Fig. 3.

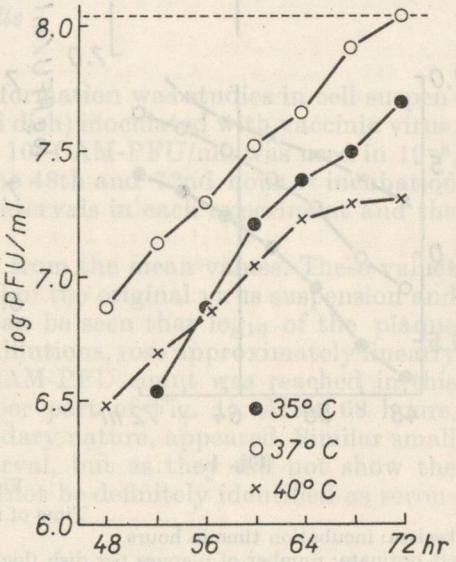

Fig. 4.

Fig. 3.

Number of cells and time of incubation

Abscissa: time of incubation in hours; ordinate: $\log_{10}$ PFU/ml orig. virus suspension

●: 4×10^6 cells/Petri dish

○: 6×10^6 cells/Petri dish

×: 8×10^6 cells/Petri dish

----------: $\log_{10}$ CAM-PFU/ml orig. virus suspension (reference)

Fig. 4.

Temperature of incubation

For explanations see Fig. 3.

containing 6×10^6 and 8×10^6 cells per dish, the reference titre was reached after 65 and 72 hours respectively.

Temperature

In the last experiment we studied plaque formation at 35, 37 and 40° C (Fig. 4). It can be seen that plaque formation at 35° C was delayed by about 8 hours as compared with plaque formation at 37° C. This delay was presumably due to lower metabolic activity of the cells since the curves for plaque formation at 35° C and 37° C are parallel. At 40° C, however, as time progres-

Table 1. Andersen and Larsen's technique. Effects of cell concentration

Exp. No.	Number of cells per dish		
	4×10^6	6×10^6	8×10^6
1	19.0*	34.5	39.0
2	19.8	27.0	33.4
3	24.7	30.5	41.0
$\bar{x}$	21.1	30.6	37.8
PFU/ml	6.33×10^7	9.18×10^7	1.13×10^8

* Average plaque count of 4 Petri dishes.

ses, the curve for plaque formation steadily diverges from the course of the curves at 35° C and 37° C. We therefore conclude that the delay in plaque formation at 40° C was due to mechanisms qualitatively different from those causing the delay at 35° C. The process of the formation of vaccinia virus plaques "in liquid medium" thus depends on the incubation temperature, the optimal temperature being 37° C.

Table 2. Andersen and Larsen's technique. Effects of incubation temperature

Exp. No.	Temperature of incubation		
	35° C	37° C	40° C
1	32.4*	39.0	10.0
2	30.0	32.0	6.0
3	41.0	50.0	14.0
$\bar{x}$	34.4	40.3	10.0
PFU/ml	1.03×10^8	1.21×10^8	3.0×10^7

* Average plaque count of 4 Petri dishes.

Andersen and Larsen's technique

Andersen and Larsen (1966) described a technique for the titration of vaccinia virus which combines the advantages of inoculation of a chick embryo cell suspension with those of an agar layer, in which the formation of secondary plaques is eliminated.

In our experiments we incubated suspensions containing 4, 6 and 8 million cells per dish inoculated with vaccinia virus suspension diluted $10^{-5.48}$ in amounts of 0.1 ml/dish. After 20 hours' incubation at 37° C, the medium

Table 3. Andersen and Larsen's technique. Effects of $NaHCO_3$ concentration

Exp. No.	% $NaHCO_3$			
	0.05	0.10	0.20	0.30
1	36*	39	41	33
2	32	36	36	29
3	36	43	40	34
$\bar{x}$	34.6	39.3	39.0	32.0
PFU/ml	1.04×10^8	1.18×10^8	1.17×10^8	9.6×10^7

* Average plaque count of 4 Petri dishes.

was decanted, the cultures were overlaid with agar medium and incubated further at 37° C; the number of plaques was read 3 days later. Five Petri dishes were used for each cell concentration.

It can be seen from Table 1 that in this case the number of cells also influenced the titration results; with 8×10^6 cells per dish the number of lesions corresponded to the titre determined on chick CAM (1.1×10^8 CAM-PFU/ml), while the lower cell concentrations gave a smaller number of lesions.

Table 4. Andersen and Larsen's technique. Effects of time of adding agar overlay

Exp. No.	Time in hours before adding agar overlay		
	20	24	28
1	32.7*	35.2	45.0
2	36.5	37.0	42.0
3	30.7	34.5	39.7
$\bar{x}$	33.3	35.6	42.0
PFU/ml	9.99×10^7	1.06×10^8	1.26×10^8

* Average plaque count of 4 Petri dishes.

The influence of the incubation temperature (Table 2) was similar to that in titration "in liquid medium". The optimal temperature was again 37° C; at 35° C the plaque count was lower and at 40° C plaque formation was significantly decreased.

The $NaHCO_3$ concentration (Table 3) within limits of 0.05 to 0.2% did not influence plaque formation. A 0.3% concentration had a slightly depres-

sant effect, no doubt because it impaired the physiological activity of the cells in the culture.

When adding the agar layer later than 24 hours after the onset of incubation in liquid medium, the increase in the number of plaques, as seen from Table 4, was accounted for by the formation of satellite secondary plaques.

Discussion

The direct inoculation of a cell suspension in Petri dishes (Andersen and Larsen, 1966) or in tubes (Slonim and Hulenová, 1966) with vaccinia virus is a technically simple method for titration according to the PFU count or TCD_{50}. In addition, as distinct from inoculation of a preformed monolayer, it allows rapid and complete adsorption of the virus.

The specific type of microepidemicity of vaccinia virus (Nishmi and Keller, 1962) permits the formation of isolated plaques of this virus "in liquid medium" and its titration according to the PFU count, without adding a gel layer; the majority of techniques of this type are based on the experiences of Postlethwaite (1960).

Our study shows that the results of titration "in liquid medium" are determined by a highly dynamic system which is difficult to standardize. In the first place, in the curve of plaque formation we observed no plateau at which the number of plaques remained constant for at least some time (Lindenmann and Gifford, 1963). It can be seen that the number of plaques increases continuously with the time of incubation, so that the time at which the results are read can be determined only in relation to the plaque count in another, known reference system. Determination of this time from the time of the appearance of secondary plaques is unreliable, not only because of the difficulty of distinguishing secondary plaques from small primary plaques, but also because the time of the formation of secondary plaques might vary according to the genetic properties of different strains of vaccinia virus (Lindenmann and Gifford, 1963), making it impossible to fix a generally valid time.

The number of cells in the system was also found to be an important factor in the rate of plaque formation. It is known (Nishmi and Keller, 1962) that vaccinia virus spreads by infiltrating from one cell into another and that relatively little is released extracellularly. We therefore assume that the greater the number of cells present over a given area critical for visibility of the plaques, the longer will be the time required for their progressive infection and destruction. The rate of formation of the visible plaque is thus a function of the number of cells per area unit in the culture. Another, equally significant, factor is the incubation temperature, which, as we have shown, can also influence the rate of plaque formation.

In our experience, titration by Andersen and Larsen's (1966) technique is the most satisfactory method for plaque titration of vaccinia virus. It has the advantage that it precludes prolonged and incomplete adsorption of the virus, which is one of the drawbacks of inoculation of a cell monolayer, and that it limits the formation of secondary plaques by the addition of an agar

layer in the early phase of virus multiplication. Here again the number of plaques is influenced by the cell concentration in the culture.

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