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STUDY OF PLAQUE TITRATION OF VACCINIA VIRUS IN CHICK EMBRYO CELL CULTURES. I. ASSAYS UNDER AN AGAR OVERLAY

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Summary. — The vaccinia virus titre determined by the plaque forming unit (PFU) count in chick embryo cell (CEC) monolayer cultures preformed in Petri dishes, then inoculated with the virus and maintained under an agar overlay was about one $\log_{10}$ unit lower than the pock forming unit (CAM-PFU) count in chick embryo chorioallantoic membranes (CAM). Vaccinia virus was adsorbed slowly onto a CEC monolayer, a steady state not being reached for six hours. The number of vaccinia virus plaques was directly correlated to the number of cells seeded into the Petri dish. For the given time of adsorption and the given culture area, the number of vaccinia virus plaques was indirectly correlated to the volume of the inoculum. At 35° and 37° C, no difference was found in the number of plaques, but at 40° C the plaque count was about 0.7 $\log_{10}$ unit lower. Within limits of 0.05—0.2%, the $NaHCO_3$ concentration did not affect plaque formation. The lower plating efficiency of vaccinia virus in CEC cultures was not due to lower sensitivity of CEC cultures to vaccinia virus.

Introduction

The development of plaques and the titration of vaccinia virus activity according to the PFU count in CEC monolayer cultures have repeatedly been studied by various authors, using different techniques and with different results (Noyes, 1953; Postlethwaite, 1960; Porterfield and Allinson, 1960; Lindenmann and Gifford, 1963; Andersen and Larsen, 1966).

The aim of this part of our work was to study the influence of a number of factors on the sensitivity and reproducibility of plaque titration of vaccinia virus infectious activity in primary CEC monolayer cultures in Petri dishes.

Materials and Methods

Cell cultures. Cells obtained by trypsinization of the bodies of 10- to 11-day chick embryos were suspended in a given concentration in growth medium, pipetted into plastic Petri dishes (NUNC, Denmark) of 5 cm diameter, and incubated for 3 days at 37° C in air containing 3—5% CO_2. The resultant monolayers were washed with phosphate buffered saline (PBS; Dulbecco and Vogt, 1954) and inoculated with virus. After adsorption of the virus for a given time at 22—24° C (3—5% CO_2), the cultures were covered with an agar layer and incubated further

at 37° C (3—5% CO_2) for four days. For reading the results, 0.7 ml neutral red solution (1 : 1,000) was pipetted on to the agar and when the monolayer was stained, characteristic plaques were counted under a magnifying-glass.

Growth medium: medium 199 (as modified by Slonim *et al.*, 1960) — 100 ml; heated calf serum — 2 ml; 7.5% $NaHCO_3$ solution — 1.4 ml; penicillin — 100 units/ml; and streptomycin — 100 μg/ml. It was used in amounts of 4 ml per Petri dish.

Agar layer: medium EPL (Michl, 1961), doubly concentrated — 100 ml; 7.5% $NaHCO_3$ solution — 2.8 ml; 2% aqueous Difco-Bacto agar solution — 100 ml; penicillin — 100 units/ml; and streptomycin — 100 μg/ml. It was used in amounts of 5 ml per Petri dish.

Titration on chick CAM. The CAM-PFU/ml values determined on the CAM of 12-day chick embryos (Slonim *et al.*, 1967) acted as references.

The vaccinia virus employed in the experiments was the strain "Praha" used in Czechoslovakia for smallpox vaccination since 1892, maintained by passages in calf skin, lyophilized in 5% peptone, and containing 1.1×10^8 CAM-PFU/ml.

Diluent: PBS (Dulbecco and Vogt, 1954) without Ca and Mg, containing 0.1% bovine albumin, 100 units penicillin/ml and 100 μg streptomycin/ml.

Results

Time of reading the results

Cells were seeded into Petri dishes in amounts of 6×10^6. The resultant monolayer was inoculated with 0.1 ml vaccinia virus suspension diluted $10^{-4.6}$. After 2 hours' adsorption, an agar layer was poured over the cultures. The number and size of the plaques was determined on each day of incubation in five dishes.

As seen from Table 1, no plaques could be detected after 24 hours of incubation. They appeared for the first time on day 2 and then increased in size and number up to day 4, when their number stopped increasing. It is therefore evident that the PFU count cannot be determined before

Table 1. Time of reading of the results

Days after inoculation	Number of plaques					$\bar{x}$	Plaque diam. mm	log PFU	Diff.
1	0	0	0	0	0	—	—	—	
2	10	8	9	10	9	9.2	1	6.57	−1.47
3	10	14	13	13	13	12.2	1—2	6.69	−1.35
4	19	21	19	28	19	21.2	1—2	6.93	−1.11
5	22	24	19	21	22	21.6	2—4	6.94	−1.10
6	20	20	22	24	21	21.4	3—4	6.93	−1.11

$\bar{x}$ = Average number of plaques per dish.
log PFU = Number of PFU per ml of the original virus suspension ($\log_{10}$).
Diff. = $\log_{10}$ PFU − $\log_{10}$ CAM-PFU.

the 4th day after inoculation. The final count of the number of plaques after the 4th day of incubation ($10^{6.93}$/ml) was 13 times smaller than the CAM-PFU count determined for the same virus suspension ($10^{8.04}$/ml). Repeated titration showed that the low PFU value in CEC cultures was reproducible.

Table 2. Time of virus adsorption

Hours of adsorption	Number of plaques				$\bar{x}$	log PFU	Diff.
1	25	33	31	29	29	7.06	−0.98
2	33	42	34	40	37	7.17	−0.87
3	42	40	45	42	42	7.23	−0.81
4	50	56	52	52	52	7.32	−0.72
5	67	64	—	—	65	7.41	−0.63
6	68	62	71	—	67	7.44	−0.60
7	—	—	—	—	—	—	—

— = Cultures had degenerated.
For other explanations see Table 1.

Time of adsorption of virus

Cell monolayers grown from 6×10^6 cells/dish were inoculated with 0.1 ml volumes of a $10^{-4.6}$ virus suspension. Every hour after inoculation, for seven hours, an agar layer was poured over groups of four dishes, which were then incubated at 37° C.

The plaque counts determined after 4 days of incubation (Table 2) confirmed the known fact that the adsorption of vaccinia virus to cell cultures is very slow and incomplete. It can be assumed that adsorption of the virus, under the given conditions, reached maximum values between the 5th and

Table 3. Number of seeded cells

Exp. No.	Cell number (in millions) per Petri dish				
	2	4	6	8	12
1	12.0*	52.0	77.2	81.0	106.8
2	10.0	35.0	50.0	75.0	102.5
3	11.2	50.5	62.5	93.0	113.0
4	12.5	45.6	78.4	85.6	108.2
$\bar{x}$	11.4	45.7	67.0	83.6	107.6
log PFU	6.66	7.26	7.43	7.52	7.63

* Average plaque count from 4 Petri dishes.
For other explanations see Table 1.

6th hour. If the time of adsorption was prolonged from 1 to 6 hours, the number of plaques was roughly doubled. Adsorption for more than 4 hours was sometimes accompanied by degeneration of the cells; after 7 hours all the cultures degenerated, despite the fact that humidity and the composition of the atmosphere (CO_2) were maintained the same. Comparison with the CAM-PFU count ($10^{8.04}$/ml) showed, however, that even 6 hours did not

ensure adsorption of all the infectious particles present in the inoculum as the number of plaques in this experiment was still four times lower after 6 hours' adsorption than in titration on CAM.

Number of seeded cells

Cells seeded in graded numbers from 2×10^6 to 12×10^6 per dish were cultivated for 3 days and the resultant monolayers were inoculated with 0.1 ml virus suspension diluted $10^{-4.6}$. After 2 hours' adsorption they were covered with an agar layer and the results were read after 4 days' incubation. The experiment was repeated four times. In attempts to prepare cultures from more than 12×10^6 cells per dish, the cells frequently degenerated and sometimes did not grow at all; 12×10^6 cells per dish was the maximum number from which a monolayer was always obtained.

Table 4. Inoculum volume

Exp. No.	Inoculum volume in ml			
	0.05	0.10	0.20	0.40
1	10.7*	16.4	20.6	40.6
2	15.2	22.8	29.0	47.8
3	12.5	22.4	28.1	36.8
4	15.7	23.2	36.4	49.0
$\bar{x}$	13.5	21.2	28.5	43.5
log PFU	7.43	7.33	7.15	7.03

* Average plaque count from 4 Petri dishes.
For other explanations see Table 1.

The results of these experiments are presented in Table 3. It is evident that, under the given conditions, the number of plaques rose together with the number of seeded cells. The greatest difference in the number of plaques was between 2×10^6 and 4×10^6 seeded cells. In cultures grown from 4×10^6 to 12×10^6 cells, $\log_{10}$ of the plaque count rose approximately linearly with $\log_{10}$ of the number of cells. On raising the number of cells from 4×10^6 to 8×10^6, the number of plaques was roughly doubled. In further experiments we therefore used 8×10^6 cells per dish.

Inoculum volume

Monolayers grown from 8×10^6 cells per dish were inoculated with graded volumes of virus suspension diluted 10^{-5}; after 2 hours' adsorption they were covered with an agar layer and the plaque counts were read after 4 days' incubation.

The results of four experiments are given in Table 4. The titre expressed as the PFU count per ml of the original virus preparation fell with increasing the volume of inoculum used for titration. Log_{10} of the titre was in a linear correlation to $\log_{10}$ of the inoculum volume; a fourfold increase in the inoculum volume produced only a twofold increase in the plaque count.

Table 5. Incubation temperature after inoculation

Exp. No.	Temperature of incubation		
	35° C	37° C	40° C
1	35.8*	40.0	5.0
2	42.5	39.2	10.2
$\bar{x}$	39.1	39.6	7.6
log PFU	7.07	7.08	6.36

* Average plaque count from 4 Petri dishes.
For other explanations see Table 1.

Incubation temperature after inoculation

Monolayers grown from 8×10^6 cells per dish were inoculated with 0.1 ml virus suspension diluted $10^{-3.48}$. After 2 hours' adsorption, the dishes were covered with agar and divided into three groups, which were incubated at 35°, 37° and 40° C respectively.

As seen from the results given in Table 5, the plaque counts at 35° C and 37° C were the same; at 40° C, however, the PFU count was about four times lower.

Table 6. Concentration of sodium bicarbonate

Exp. No.	% $NaHCO_3$			
	0.05	0.10	0.20	0.30
1	34.0*	42.5	32.2	29.7
2	36.7	39.0	39.7	12.6
$\bar{x}$	35.3	40.7	35.9	21.1
log PFU	7.03	7.09	7.03	6.80

For explanations see Table 5.

Amount of $NaHCO_3$

The conditions were the same as in the preceding experiment, except that only one incubation temperature (37° C) was used and that the agar overlay contained graded amounts of $NaHCO_3$ as shown in Table 6.

Within limits of from 0.05—0.2%, the $NaHCO_3$ concentration did not affect the plaque count; 0.3% $NaHCO_3$ lowered plaque formation, while 0.4% led to general degeneration of the cells in the monolayer.

Discussion

According to the above results, the vaccinia virus titre determined by the PFU count on a CEC monolayer is about 10 times lower than the titre determined by the pock count on chick embryo CAM. Other authors had similar experiences; for example, Noyes (1953) quoted values six times lower; Porterfield and Allinson (1960), using $NaHCO_3$ buffer, gave values up to 10 times lower; and Postlethwaite (1960), in titration in liquid medium, found values 4—5 times lower. Lower titres were also found by Youngner (1956) in monkey kidney cells and by Galasso and Sharp (1964) in L cells. It can therefore be assumed that the lower PFU values in CEC cultures are not due to specifically lower sensitivity of this type of cells. Our findings also confirmed this conclusion.

In the present study we verified the slow adsorption of vaccinia virus described by most of the authors cited above. Prolongation of the time of adsorption from 1—2 to 5—6 hours doubles the number of plaques. Slow adsorption of the virus is also undoubtedly the cause of slow, successive development of the number of lesions; between the 2nd and 3rd day, the number of visible lesions rises by about 30%, and between the 3rd and 4th day by about 70%, while from the 4th day onwards it remains unchanged.

The number of seeded cells from which the monolayer was formed was found to be another factor influencing the number of lesions. An increase in the number of seeded cells was accompanied by an increase in the number of plaque. Within limits of from 4×10^6 to 12×10^6 cells the increase in the plaque count is almost linear; with 2×10^6 seeded cells per dish, however, the number of plaques is much lower than the anticipated number (i.e. from a linear relationship). The reason is no doubt that monolayers grown from 2×10^6 cells are so thinly distributed that in some cases the virus is not adsorbed onto sensitive cells, or that plaques are formed but are hard to detect. From 4×10^6 cells per dish, however, coherent monolayers are formed, while the plaques are easily discernible and cannot be missed. When the number of seeded cells was raised from 4×10^6 to 8×10^6 per dish, the number of lesions rose by 82% and on raising it from 4×10^6 to 12×10^6 per dish the increase in the number of lesions was 134%. At present we are unable to explain this phenomenon and we likewise do not know whether it occurs with other virus species. In the case of vaccinia virus, however, it can be assumed that it also applies in other cell systems, since Geme (1963) reported that on doubling the number of cells in a culture of a human liver cell line the number of plaques formed by vaccinia virus was likewise doubled.

The results of the present study further show the existence of an inverse correlation between the volume of inoculum and the number of plaques; in $\log_{10}$ values the relationship is linear. The minimum volume used (0.05 ml/dish) gave the relatively highest virus titre. These results are in agreement with observations made in titration of vaccinia virus on chick embryo CAM (Slonim *et al.*, 1967) and are no doubt of general significance, as they are also in agreement with those of Fiala and Kenny (1966), who

found an inverse correlation between the inoculum volume and the plaque count in titration of rhinoviruses in HeLa cells. As far as the explanation of this phenomenon is concerned, it can be assumed that an increase in the volume of the inoculum reduces the possibility of the virus particles encountering sensitive cells over a given area of the monolayer, with the result that the number of plaques falls.

The influence of a temperature of 40° C is manifested by a distinct decrease in the plaque count; this is already generally known (Kirn *et al.*, 1965). The influence of the amount of $NaHCO_3$ is the same within limits of from 0.05—0.2%; from 0.3% upwards, the number of plaques falls, no doubt as a result of direct injury to the cells in the monolayer.

The present study indicates that the number of seeded cells, the volume of the inoculum and the incubation temperature, as well as the time of reading the results and the time of adsorption, are factors which can influence the vaccinia virus PFU value in CEC monolayers preformed in Petri dishes. In addition, the present and previous (Slonim and Hulenová, 1966) results show that the low plating efficiency of vaccinia virus is not due to low sensitivity of the CEC cultures used.

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