A Simple *in vitro* Assay for Vaccinia Virus Using Tube Cultures of Human Cells.* (28298)

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The superiority of plaque assay over 50% infectivity titrations for *in vitro* quantitation of animal viruses is well established(1). Vaccinia virus manifests a distinctive focal type of cytopathology *in vitro* due most likely to a slow process of virus dissemination from infected to neighboring cells(2). Using only a fluid overlay maintenance medium and appropriate timing one may observe distinct plaques. This cultural phenomenon provides an accurate, quantitative system of plaque assay in the absence of agar(3). The method may also be adapted to tube cultures in lieu of the more commonly used bottle cultures.

Materials and methods. An established line of liver-derived human cells (strain Lass)(4), originally cultivated in the presence of human serum, was adapted to rapid growth in a medium containing Hanks' balanced salt solution (BSS), 70 volumes; 1% yeast extract, 10 volumes; calf serum, 20 volumes; penicillin, 100 units/ml; and streptomycin, 100 μg/ml. For experimental purposes, cells were maintained in a medium consisting of BSS, 85 volumes; 1% yeast extract, 10 volumes; calf serum, 5 volumes; and antibiotics as above. Cell inoculum per 16 $\times$ 125 mm screw-cap tube was approximately 200,000 cells per ml of growth medium. After 48 hours of incubation at 37°C tube cultures could be used for plaque assay.

Vaccinia virus seed used for these experiments was prepared in HeLa cells and subsequently in strain Lass cells, both virus pools titering $10^{5.7}$ 50% tissue culture infective doses ($TCID_{50}$) per ml. The virus originated as avianized smallpox vaccine prepared for commercial use by Lederle Laboratories. Virus dilutions were made in BSS.

After appropriate intervals of incubation at 37°C tube cultures, previously inoculated with vaccinia virus, were emptied of maintenance medium and stained with 1.0 ml of crystal violet (0.05%) for 10 to 20 minutes (5). A Wolf table top illuminator† was used to facilitate macroscopic enumeration of vaccinia virus plaques.

Results. Fig. 1 illustrates the discrete nature of the plaque foci in tube cultures. Plaques vary from approximately 0.5 mm to 1.5 mm in size.

Inoculation of Virus. Tube cultures were inoculated with stock virus, diluted 10^{-2}, by two techniques: (a) adsorption of 0.1 ml at 37°C for one hour, tilting the cultures every 15 minutes to allow the inoculum to flow completely over the monolayer, followed by addition of maintenance medium, 1.0 ml, and incubation; (b) simple addition of 0.1 ml to cultures containing maintenance medium, 1.0 ml, vigorous agitation for one minute, and incubation for 48 hours. Allowing virus inoculum to adsorb to monolayers for one hour in absence of maintenance medium yielded almost twice the number of plaques observed in cultures inoculated directly. Prolonged adsorption for 2 and 3 hours failed to enhance plaque yield.

Linearity and Precision of Assay. The linear relationship between number of plaques and virus concentration was established by preparing 2-fold dilutions of stock virus, $10^{-2.0}$ through $10^{-3.2}$, then adding 0.1 ml aliquots of each dilution to 8 replicate tube cultures containing maintenance medium, 1.0 ml. In Fig. 2 may be seen the linear curve described when the mean of plaque counts per replicate culture is plotted against relative virus concentration. Table I demonstrates the greater reliability of data obtained when approximately 50 plaques occur per tube culture.

Effect of Cell Population on Plaque Production. To replicate cultures, shown by trypsinization and hemocytometer count to contain either 175,000 or 360,000 cells per tube,

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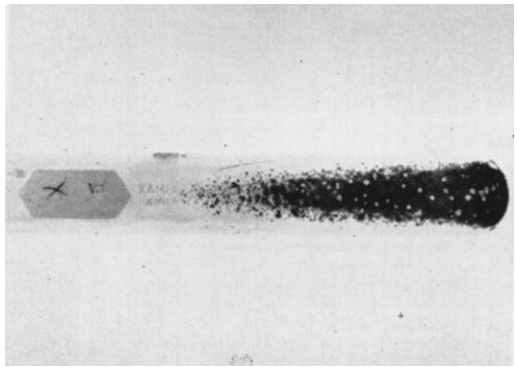

FIG. 1. Plaques of vaccinia virus on strain Lass cells in 16 × 125 mm culture tube. Stained with 0.05% crystal violet at 48 hr. Magnification .75×.

was added 0.1 ml of virus diluted 10^{-2}. After 48 hours of incubation cultures containing the larger cell population yielded twice the number of plaques observed in tubes with 175,000 cells.

Optimal Time for Staining Plaques. Replicate cultures containing 1.0 ml of maintenance medium were inoculated with 0.1 ml of stock virus diluted $10^{-2.3}$. By counting plaques on consecutive days an approximate 4-fold increase in single plaque foci occurred between the first and second day of incubation (Table II). By the third day additional increments could be attributed to formation of secondary plaques. This pattern of secondary plaque formation adjacent to the initial plaque foci persisted through the next 48 hours. From the third through the fifth day there was also an increase in plaque size.

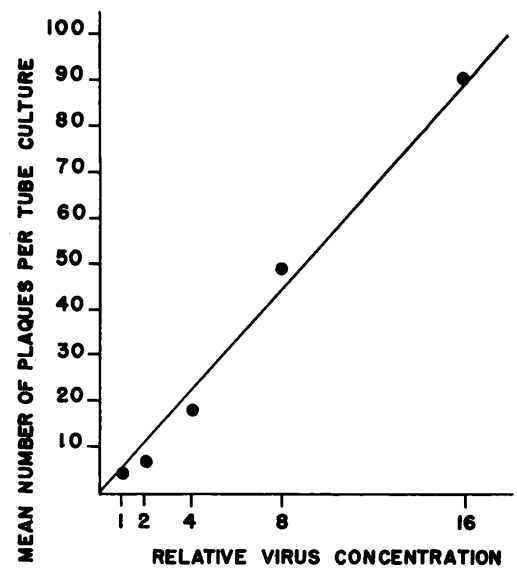

FIG. 2. Linear relationship between number of plaques and virus concentration.

Comparison of Fluid and Agar Overlay Technique. A 0.1 ml inoculum of virus, diluted 10^{-2}, was adsorbed to each of several replicate cultures for one hour at 37°C followed by addition of maintenance medium, 1.0 ml, to half the tubes and 0.6% agar medium (1 part 3% Difco agar plus 4 parts maintenance medium), 1.0 ml, to the remainder. A striking difference in plaque yield was noted in cultures incubated for 48 hours with fluid (mean = 113) as compared with soft agar overlay (mean = 37). Not only were fewer plaques observed under agar but plaques were less distinct and smaller than with the fluid overlay technic. The experiment was repeated with the added observation of the evolution of plaque foci under the agar overlay for 96 hours. Even under soft agar the increment in plaque formation at 72 hours was due to appearance of secondary plaques. At 72 hours plaques under soft agar were more distinct

TABLE I. Effect of Incremental Concentrations of Virus on Variation of Plaque Formation in Replicate Tube Cultures Using Fluid Overlay.

Log_{10} dilutions of virus inoculum (0.1 ml/ tube culture)	No. of plaques per replicate tube culture at 48 hr	Mean and standard deviation of plaques per tube culture
$10^{-2.0}$	120, 74, 97, 90, 87, 70, 88, 90	90 ± 10.6
$10^{-2.3}$	46, 49, 48, 40, 53, 47, 53, 55	49 ± 2.8
$10^{-2.6}$	12, 14, 16, 20, —, 19, 19, 28	18 ± 5.9
$10^{-2.9}$	6, 5, 7, 5, 6, 8, 12, 8	7 ± 2.5
$10^{-3.2}$	4, 4, 6, 7, 6, 4, 5, 6	5 ± 2.5

TABLE II. Serial Daily Observation of Plaque Formation Using Fluid Overlay, Virus Inoculum 250 $TCID_{50}$/0.1 ml/tube culture.

Day of observation	Mean of plaque number per tube culture	Secondary plaques
1	14	none
2	58	"
3	89	present
4	111	"
5	121	"

and more easily counted. By the fourth day, in contrast to cultures with fluid overlay, monolayers under soft agar begin to peel extensively.

Discussion. The data support Postlethwaite's conclusion that a fluid overlay method for plaque assay of vaccinia virus provides a very satisfactory quantitative system(3). Adsorption of virus inocula on the culture monolayers for one hour permits maximum recovery of virus particles from experimental fluids. For comparative experiments, however, simple addition of inocula to tube cultures provides adequate quantitative data.

The linear relationship between number of plaques and virus concentration with fluid overlay implies that a single virus particle may be recovered in this system as a single plaque focus. Such a statement is true if one accepts the theories of Dulbecco(1), although subject to modified interpretation for vaccinia virus in light of the experiments of Galasso and Sharp(6). One might conclude that there is a linear relationship between aggregates of infectious particles, large or small, and plaque foci.

In the easily counted range of approximately 40 to 50 plaques per tube culture the precision of the assay is quite adequate. When the number of plaques per culture falls below 20 a considerable loss in accuracy occurs.

The difference in plaque yield realized when the cell population of the tube culture is altered 2-fold lends support to the concept of a Poisson distribution of infectious particles as championed by Dulbecco(1). With the larger cell population accrues a greater recovery of plaque-forming particles per given virus inoculum. In addition, an initial cell inoculum of 200,000 cells per tube culture provides a confluent monolayer after only 48 hours of incubation at 37°C. A smaller cell inoculum may require 3 to 4 days before an adequate monolayer is obtained.

It is apparent from the data that meaningful and accurate plaque counts may be made in 2 days. Subsequent incubation serves only to distort virus quantitation because of progressive infection of contiguous cells with formation of secondary or daughter plaques. This was true even when a soft agar overlay was employed.

In comparative assay experiments the fluid overlay technique yielded a greater number of vaccinia virus plaques than the soft agar overlay method. These plaques were also larger and more easily counted. The additional steps of melting agar, prewarming maintenance medium, diluting agar in medium to appropriate osmolarity; the necessity for rapid pipetting of such medium into culture tubes; and the observation of extensive disruption of the cell sheet upon 72 and 96 hours of incubation under agar punctuate the technical disadvantages of the soft agar overlay method. It is possible that the agar inhibitors described by Takemoto and Liebhaber(7) may explain the reduction in number and size of plaques in tube cultures overlaid with soft agar. This possibility remains unproven, however, for this assay system. With these observations and comments in mind the advantages and simplicity of the fluid overlay system would seem obvious.

The use of tube cultures, containing 1.0 ml of cells, in lieu of larger prescription or plaque bottles, containing 5.0 ml of cells, allows one to execute an economic program for continuous cultivation of stock cells in preparation for experiments involving plaque assay of vaccinia virus.

Summary. A satisfactory quantitative plaque assay of vaccinia virus may be obtained in tube cultures of established human cells using a simple method of fluid overlay. With this method stock cells may be employed with economy and the technical disadvantages of an agar overlay circumvented.

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