Comparison of the Immunofluorescent-Cell Counting and Plaque Methods for the Assay of Vaccinia Virus

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The immunofluorescent-cell counting method was compared with the agar- and liquid-overlay plaque techniques for the assay of vaccinia virus. In addition to being as quantitative, reproducible, and simple to use as the two plaque techniques, the immunofluorescent assay method was found to be more sensitive, rapid, and specific.

Quantitative assays of infective virus particles based on the enumeration of fluorescent cells containing viral antigen have been developed and standardized for only a limited number of viruses representing a few major groups, e.g., paramyxoviruses (11), adenoviruses (7), herpesviruses (2), reoviruses (10), and poxviruses (1, 3). In assays of these viruses, the immunofluorescent-cell counting (ICC) technique was found to be highly sensitive, quantitative, reproducible, specific, and rapid.

Although the ICC technique has been applied to poxviruses (1, 3, 10), a comparison with the equally quantitative, sensitive, and reproducible liquid- and agar-overlay plaque techniques routinely used for vaccinia virus assay (6, 8) has not been made. This report describes the development and standardization of the ICC technique for the quantitative assay of vaccinia virus and compares it with the liquid- and agar-overlay plaque techniques.

MATERIALS AND METHODS

Virus. The stock of vaccinia virus used in all experiments was passaged in HeLa cells (10 times) and in BS-C-1 (African green monkey kidney) cells (4 times). It contained approximately $2 \times 10^4$ to $2.5 \times 10^4$ plaque-forming units (PFU) per 0.1 ml as determined by the liquid-overlay plaque technique (6).

Cells. Monolayers of BS-C-1 cells were grown in Eagle basal medium (EBM) containing 5% fetal calf serum (FCS). These cells were used for both plaque and ICC techniques.

Plaque assay. Infectious virus was assayed in monolayers of BS-C-1 cells grown in Leighton tubes (L tubes) by the liquid-overlay plaque technique of Nishimi (6). Briefly, the monolayers were washed with three 1-ml portions of Hank's balanced salt solution (BSS) and infected with 0.1-ml volumes of any virus dilution (four tubes/dilution). After an adsorption period of 2 hr at 37 C, at which time virus adsorption reached an equilibrium (4), the unadsorbed virus was removed by washing the cultures with three 1-ml portions of BSS, and 1 ml of EMB-0.1% FCS was added. In certain experiments, an agar overlay (0.6%) containing EMB-0.1% FCS was added and removed before enumeration of plaques. With both types of overlays, observation of the plaques formed was facilitated by staining with 0.01% crystal violet for 5 min, followed by a wash with BSS. The number of plaques was then determined under a dissecting microscope.

Uninfected Leighton tube cultures mock-infected with experimental medium and processed in a fashion similar to that described for the infected cultures served as controls. Control cultures did not exhibit any cytopathology during the period examined.

ICC technique. No. 1 round cover slips (15 mm) in sterile Falcon tissue culture dishes (90 by 15 mm) were each seeded with $3 \times 10^4$ to $5 \times 10^4$ cells. After the cells were allowed to attach to the cover slip for 1 hr at 37 C in a CO2 incubator, 2 ml of EMB-5% FCS was added, and the cultures were reincubated. Monolayers of cells were formed in 48 to 72 hr and were infected with 0.02 ml of any virus dilution (four cover slips/dilution). After adsorption for 2 hr at 37 C, the unadsorbed virus was removed by washing the cover slips with three 1-ml portions of BSS, and EMB-0.1% FCS was added. At the end of the appropriate incubation period, the cover slips were washed with cold BSS, fixed in cold acetone (10 min), air-dried, and stored at 4 C. The cover slips were stained with fluorescein isothiocyanate (FITC)-conjugated immune rabbit gamma globulin against vaccinia virus as described previously (5) and were examined under a Zeiss fluorescence microscope. The number of fluorescent cells on the entire cover
slip was determined. To calculate the number of immunofluorescent units of virus per milliliter, the average number of fluorescent cells per cover slip was multiplied by the reciprocal of the dilution of virus inoculum and a volume factor (for conversion to milliliters).

Uninfected cover slips mock-infected with experimental medium and processed in a fashion similar to that described for the infected cultures served as controls.

Antiserum. Rabbit anti-vaccinia serum was obtained from the Biologic Reagents Section, National Center for Disease Control, Atlanta, Ga. The preparation of the immune globulin and the conjugation with FITC have been described (5). The specificity of the staining by labeled globulin was determined by the blocking type of experiment with unlabeled and labeled antibody preparations and by the staining of uninfected cover slips.

RESULTS

Plaque development and assay. The liquid- and agar-overlay techniques for the titration of vaccinia virus were compared. With both techniques, pinpoint plaques were visible as early as 36 hr after infection, and by 48 hr the plaques were about 1 mm in diameter. Although by 72 hr the plaques had further increased in size, the number remained essentially unchanged. At 96 hr postinfection, there was an increase in the number of plaques formed only in the liquid-overlay tubes. Because of their small size, these probably represented secondary plaques. These results indicated that the optimal incubation period for the enumeration of plaques for both types of overlays was 48 hr.

A quantitative evaluation of the two plaque methods was made, and a linear relationship was demonstrated between virus concentration and the number of plaques formed for both types of overlays (Fig. 1). The data, representative of four experiments, suggested that each plaque was the consequence of infection by a single infective virus particle. The virus titers obtained for both types of overlays were essentially similar.

Immunofluorescence assay. A previous study on the development of vaccinia antigens indicated that, although cytoplasmic fluorescence in the infected cell could be detected as early as 6 hr postinfection, such fluorescence could be readily discerned at 12 hr after infection (4). The present experiment was designed to establish the optimal period for the enumeration of readily discernible fluorescing cells resulting from a single cycle of infection. Coverslip cultures of HeLa cells were infected with vaccinia virus at a multiplicity of 1, and the number of infected cells was determined at various periods after infection. The number of fluorescing cells was found to be constant between 12 and 18 hr after infection. Beyond 20 hr postinfection, the number of fluorescing cells increased. At these later periods, the foci which began as single fluorescent cells became enlarged to involve groups of two to five cells. Also, the cells exhibited various degrees of fluorescence. Based on these observations and findings, the optimal period for counting fluorescing cells was established at 18 hr.

A quantitative evaluation of the fluorescent-cell counting procedure was made, and a linear relationship was demonstrated between virus concentration and the number of fluorescing cells (Fig. 2). As was found for the plaque techniques, the present data suggest that each fluorescent cell was the consequence of infection by a single infective particle. Thus, vaccinia
virus could be titrated by counting single cell foci at 18 hr postinfection.

The specificity of the staining by labeled globulin was indicated by the absence of fluorescing cells in uninfected cover slips. Specificity was further indicated when the blocking type of experiment with unlabeled antibody was performed. In this test, prior treatment of infected cover slips with unlabeled globulin blocked the binding of its labeled counterpart.

In other experiments, the sensitivities of the two plaque procedures and of the fluorescent-cell counting technique were compared (Table 1). The mean sensitivity of the fluorescent-cell counting technique for vaccinia virus was found to be four to five times greater than either the agar- or liquid-overlay plaque techniques. Application of Student’s t test (9) at confidence limits of 95% indicated that the differences between the plaque and the immunofluorescence techniques were statistically significant ($P < 0.01$).

Reproducibility of results is an essential feature of any assay system. All three techniques showed reproducible titers when titrations were carried out on four different occasions with the same virus pool (Table 1).

**DISCUSSION**

An ideal viral assay method should be highly sensitive, quantitative, reproducible, rapid, and simple. The present data indicate that, of the three techniques examined, titration of vaccinia virus by the ICC method best fulfills the above criteria. The liquid- and agar-overlay plaque techniques were found to be equally sensitive, quantitative, and reproducible, and relatively simple to use; both techniques, however, failed to produce easily discernible plaques until 48 hr after infection. In contrast, the ICC method, in addition to possessing all of the advantages mentioned above, was found to be more sensitive and rapid. The mean titer of vaccinia virus by the ICC method was five times greater than by either of the two plaque techniques. Furthermore, fluorescent cells could be easily enumerated within 18 hr after infection. In a study in which only a comparison of the standard agar-overlay plaque technique and the immunofluorescence method was made, Spendlove and Lennette (10) also concluded that the latter method was four times more sensitive and rapid than the former. However, to localize virus infection, the authors included in their immunofluorescence procedure a soft-agar overlay which was removed at the time of washing and fixation. In the present study, the omission of the overlay was found not to affect the accuracy of the titration. However, the importance of predetermining the optimal period, which is 18 hr postinfection, for the enumeration of fluorescing cells must be stressed. Beyond this period, an increase in the number of fluorescing cells was obtained which suggests the occurrence of a second cycle of infection. An additional advantage of the ICC method is its specificity as indicated by the blocking test and the absence of fluorescing cells in uninfected cultures. This would eliminate the possible involvement of other plaque-producing viral contaminants. Although it fulfills many of the criteria used to describe an ideal viral assay method, the ICC method has the following disadvantages: (i) a requirement for the preparation of highly specific labeled antibody to the virus, and (ii) the inability of picking clones of virus.

**LITERATURE CITED**

1. Carter, G. B. 1965. The rapid detection, titration and differentiation of variola and vaccinia viruses by a fluorescent antibody-covertlip cell monolayer system. Virology 25:659-662.
2. Goodheart, C. R., and L. B. Jaross. 1963. Human cytomegalovirus. Assay by counting infected cells. Virology 19:532-535.
3. Habon, N. 1965. Assay of variola virus by the fluorescent cell-counting technique. Appl. Microbiol. 13:865-871.
4. Loh, P. C., and F. E. Payne. 1965. Effect of 5-fluorophenylalanine on the synthesis of vaccinia virus. Virology 25:560-574.
5. Loh, P. C., and J. L. Riggs. 1961. Demonstration of the sequential development of vaccinial antigen and virus in infected cells: observation with cytochemical and differential fluorescent procedures. J. Exp. Med. 114: 149-160.
6. Nishimi, M., and R. Keller. 1962. Titration of vaccinia virus and its neutralization by the plaque technique. Nature (London) 183:150-151.
7. Phillips, L. 1961. Adenovirus assay by the fluorescent

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**Table 1. Assay of vaccinia virus by the plaque and immunofluorescent-cell counting techniques**

| Assay no. | Liquid overlay (PFU/ml) | Agar overlay (PFU/ml) | Immunofluorescent-cell counts (IU/ml) |
|-----------|------------------------|----------------------|----------------------------------|
| 1         | 2.5 x 10^5             | 2.0 x 10^5           | 9.3 x 10^4                       |
| 2         | 2.0 x 10^5             | 2.2 x 10^5           | 9.1 x 10^4                       |
| 3         | 2.4 x 10^5             | 1.8 x 10^5           | 9.8 x 10^4                       |
| 4         | 2.1 x 10^5             | 1.5 x 10^5           | 9.8 x 10^4                       |
| Mean      | 2.25 x 10^5            | 1.87 x 10^5          | 9.42 x 10^4                      |
| SD        | 0.23 x 10^5            | 0.31 x 10^5          | 0.87 x 10^4                      |
| SE        | 0.11 x 10^5            | 0.15 x 10^5          | 0.43 x 10^4                      |

* PFU = plaque-forming units; IU = immunofluorescent units.
cell-counting procedure. Virology 15:263-268.
8. Postlethwaite, R. 1960. A plaque techniques for the titration of vaccinia virus in chick embryo cell and some features of vaccinial infection in this system. Virology 10:466-482.
9. Snedecor, G. W., and W. G. Cochran. Statistical methods, 6th ed. The Iowa State Univ. Press, Ames.
10. Spendlove, R. S., and E. H. Lennette. 1962. A simplified immunofluorescent plaque method. J. Immunol. 89:106-112.
11. Spendlove, R. S., E. H. Lennette, C. O. Knight, and J. N. Chin. 1963. Development of viral antigen and infectious virus in HeLa cells infected with reovirus. J. Immunol. 90:548-553.
12. Wheelock, E. F., and I. Tamm. 1960. Enumeration of cell-infecting particles of Newcastle disease virus by the fluorescent antibody technique. J. Exp. Med. 113: 301-316.