Plaque Assay for Pneumonia Virus of Mice

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Received for publication 27 July 1970

A plaque assay for the quantitation of pneumonia virus of mice is described. To obtain reproducible plaque formation, proteolytic enzymes had to be incorporated in the overlay medium. The plaque morphology observed in the presence of pancreatin and chymotrypsin was superior to that seen with trypsin. Although the plaque assay was found to be slightly less sensitive than the TCID₅₀ determination described by Harter and Choppin, it enables cloning of the virus by plaque selection and permits further study of pneumonia virus of mice.

Until Harter and Choppin (2) propagated pneumonia virus of mice (PVM) in baby hamster kidney cells and determined the concentration of virus leading to hemagglutinin production in these cells, the only assay for infectious PVM was by intranasal inoculation in mice (3). Recently Came et al. (1) showed that certain influenza viruses which do not produce plaques in chick embryo fibroblasts do so when proteolytic enzymes are added to the overlay medium. Since PVM shares certain properties in common with the myxovirus group, the influence of proteolytic enzymes on PVM plaque formation was investigated. The present paper describes a method of determining infectious PVM by plaque assay.

MATERIALS AND METHODS

Virus. PVM was obtained in its 13th passage in BHK-21 cells from P. W. Choppin, Rockefeller University, and was passed an additional three times in BHK-21 cells followed by passage in 16-g CD-1 male mice (Charles River Breeding Laboratories, Inc., Wilmington, Mass.). The mice were infected intranasally with 10% infected mouse lung suspensions, and, 6 to 8 days after inoculation, surviving animals were sacrificed and a 10% lung suspension was prepared in Eagle’s minimal essential medium with Earle’s salts containing 1% bovine serum albumin (BSA). Virus was stored at -70 C.

Cell culture. BHK-21/C13 cells were obtained from P. W. Choppin. Growth medium consisted of BHK-21 medium (Grand Island Biological Co., Grand Island, New York) supplemented with 10% fetal calf serum and 50 µg of gentamicin sulfate per ml (Schering Corp., Bloomfield, N.J.). Petri plates (15 by 60 mm, Falcon Plastics) were prepared from monolayers which had been propagated in roller bottles (surface area, 1,100 cm²) containing 300 ml of growth medium. Roller bottles were rotated at 0.3 rev/min at 37 C, and after 3 days the growth medium was replaced with maintenance medium consisting of BHK-21 medium, 2% fetal calf serum, and 50 µg of gentamicin sulfate per ml. Subculture into petri plates was done 24 to 48 hr after addition of maintenance medium. Plates were inoculated with virus 3 days after seeding.

Hemagglutination assay. Hemagglutination titrations were performed with 0.8% mouse erythrocytes in phosphate-buffered saline (PBS) containing 0.25% BSA (2).

TCID₅₀ determination. Cell culture assays to determine the virus dilution leading to the synthesis of hemagglutinin in 50% of the cultures (TCID₅₀) were performed according to the method of Harter and Choppin (2) with slight modification as described below. Five confluent monolayers of BHK-21 cells in petri plates (15 by 60 mm) were inoculated with 0.5 ml of virus, and, after an adsorption period of 3 hr at 37 C, the inoculum was removed and 10 ml of L-15 medium (Grand Island Biological Co., Grand Island, N.Y.) containing 1% BSA was added to each plate. The presence of hemagglutinin in each dish was determined after 10 days incubation at 37 C.

Plaque assay. Monolayers were inoculated with 0.5-ml dilutions of virus in L-15 medium containing 0.5% BSA. After 3 hr of adsorption, plates were overlaid with 10 ml of L-15 medium containing 1% BSA, 0.8% agarose, and various concentrations of pancreatin or other enzymes. Plates were usually incubated for 10 days at 37 C in 5% CO₂ in humidified air and were stained with 1% iodonitrotetrazolium violet (INT) purchased from Sigma Chemical Co., St. Louis, Mo. Pancreatin (Nutritional Biochemicals Corp., Cleveland, Ohio), purified trypsin, and alpha chymotrypsin (Worthington Biochemical Corp., Freehold, N.J.) were employed in the overlay medium at final concentrations of 0.03, 0.001, and 0.001%, respectively.

RESULTS AND DISCUSSION

The morphology of PVM plaques in the presence of enzymes can be seen in Fig. 1. Plaques in the presence of trypsin-containing medium were irregular in shape, but when pancreatin or chymotrypsin was substituted for trypsin the plaques were clear, distinct, and measured 1 to 3 mm in diameter after 10 days. Under these
FIG. 1. Appearance of pneumonia virus of mice plaques in BHK 21/C13 cells. The plates were stained with 1% iodonitrotetrazolium violet 10 days after infection. (A) No enzyme, plaques (arrow) are faintly visible, (B) chymotrypsin (0.001%); (C) trypsin (0.001%); (D) pancreatin (0.03%).

TABLE 1. Effect of solidifying agent in medium on plaque numbers of PVM in BHK-21 cells

| Solidifying agent       | Avg no. plaques per plate |
|-------------------------|----------------------------|
| 0.8% Agarose            | 90                         |
| 0.8% Ionagar            | 84                         |
| 0.8% Noble Agar         | 90                         |
| 0.8% Purified Agar      | 72                         |
| 0.8% Agar (Difco)       | 91                         |

*All assays were performed with 0.03% pancreatin in the overlay medium. Without pancreatin, plaques were too indistinct to obtain accurate numbers. Each plaque count represents the average obtained from five petri plates.

conditions, the earliest time at which plaques could be visualized was 6 days after inoculation. A linear relationship between plaque number and virus dilution was obtained. Antiserum to PVM prevented plaque formation when incorporated into the overlay medium. In the absence of enzymes, poorly defined plaques could be seen after the eighth or ninth day and were less distinct,

irregular in shape, and fewer in number. Plaque numbers did not increase after 10 days, and plates were usually stained on day 10, reincubated at 37 C, and counted on day 11. Accurate plaque counts in the absence of enzyme were

TABLE 2. Comparison of various assays of PVM propagated in mice or cell culture

| Virus source       | HA* | LD50 | PFU | TCID50 |
|--------------------|-----|------|-----|--------|
| Mouse lung         | 256 | 1.3 × 10^3 | 4.0 × 10^4 | 7.0 × 10^6 |
| Cell culture fluid | 128 | <1 × 10^4   | 1.0 × 10^6 | 2.9 × 10^5 |

*Hemagglutination.

* Titer per milliliter based on arithmetic mean of two tests employing five mice per dilution for LD50, five plates per dilution for plaque-forming units (PFU), and 10 plates per dilution for TCID50.

* Ten per cent suspension of lung from fifth mouse passage.

* Fluid from 16th BHK-21 passage.
frequently impossible due to the indistinct plaque formation.

Sulfated polysaccharides contained in agar have been reported to exert inhibitory effects on myxovirus growth (4, 5), and the similarity of PVM to myxoviruses prompted experiments to determine the effect of various solidifying agents on the efficiency of plaque formation by PVM. It can be seen in Table 1 that the number of plaques which developed under various overlays was essentially the same.

Table 2 illustrates the comparison of various assays of PVM propagated in mice or cell culture. It can be seen that passage of virus in BHK-21 cells decreases the virulence of the virus for mice and that the efficiency of the plaque assay is only slightly less sensitive than the TCID$_{50}$ assay. It is more sensitive than the LD$_{50}$ determination.

Cells under enzyme-containing medium stained as intensely with INT as those in the absence of enzymes, but microscopic observation revealed that they had lost their fibroblastic appearance and separated from each other as early as 24 hr after being overlaid. However, monolayers in this condition were best suited for distinct plaque development. Concentrations of enzymes 10-fold greater than employed above destroyed the monolayers.

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