A Plaque Assay for Nuclear Polyhedrosis Viruses using a Solid Overlay

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SUMMARY

The nuclear polyhedrosis viruses of Trichoplusia ni and Autographa californica produce plaques in monolayers of Spodoptera frugiperda cells under a solid overlay containing agarose as the solidifying agent. Plaques are visible macroscopically after staining the cells with neutral red or with the tetrazolium salt, INT, and a linear dose response is observed. The sensitivity of the assay is less than that obtained using an end-point dilution technique; however, plaque formation does provide a simple means of cloning virus.

Although it has been possible to plaque vertebrate viruses for many years, assays in which macroscopic plaques are formed under agarose overlays have not been available for viruses of invertebrates until recently. The formation of macroscopic plaques in monolayers of Spodoptera frugiperda cells infected with several baculoviruses is described in a preliminary report by Knudson (1976). Macroscopic plaques were also observed in Trichoplusia ni cell monolayers, with a purified derivative of agarose (Seaplaque agarose) as the solidifying agent in the overlay (Wood, 1976). This paper gives a more detailed description of plaque formation by nuclear polyhedrosis viruses (NPV) in S. frugiperda cells under a solid overlay containing agarose.

The technique was developed using the plaque-purified MP strain of T. ni NPV at the third passage level in cell culture (T. ni MP3; Potter, Faulkner & MacKinnon, 1976). This stock was produced in S. frugiperda cells (SF) and is designated T. ni MP3 (SF). A continuous line of S. frugiperda cells, received from D. L. Knudson (Yale Arbovirus Centre, New Haven, Conn., U.S.A.) and grown in BML-TC10 medium (Gardiner & Stockdale, 1975), was used. In these experiments, the cells were between the 165th and 185th passage in culture. T. ni (TN-368) cells (Hink, 1970) were also grown in BML-TC10 medium and were beyond the 900th passage in culture.

For the plaque assay, Corning plastic Petri plates (35 mm diam.) were seeded with 10^6 viable S. frugiperda cells in 2 ml BML-TC10 medium. Viability was assessed by the trypan blue exclusion test and viable cells always accounted for more than 90% of the cell population. Following attachment of the cells (1 to 5 h at 27 °C), the monolayers were scanned. A considerable proportion of cells were in mitosis or had recently divided, indicating that the cells used in these experiments were actively growing. The medium was removed by suction with a vacuum pump and 0.1 ml virus inoculum was applied to the centre of each plate. During the 1 h virus adsorption period the plates were rocked in a humid container on a rocker platform (1 oscillation/min) at room temperature. After removal of the virus inoculum, 2 ml overlay medium containing 1.5% Seaplaque or Seakem agarose (Marine Colloids, Inc.) was added. It was prepared as follows. A 3% solution of agarose in distilled water was autoclaved and held at 37 °C in a water bath. Before use, the 3% agarose was mixed in equal proportions with LS-BML-TC10 (BML-TC10 containing inorganic salts at half the normal concentration but with all other constituents at the normal concentration)
Fig. 1. Appearance of plaques of *T. ni* NPV in *S. frugiperda* cells stained with neutral red. Overlay contained Seakem agarose.
which had been warmed to 37 °C. After the agarose had solidified, 1 ml BML-TC10 medium was added to each plate as a liquid overlay above the agarose. This addition was found to be essential for survival of the cells.

Plates were examined 5 days post-infection (p.i.) and plaques were scored unstained, with the use of the microscope. Plaques varying in diam. from < 1 to 3 mm could be seen after staining the monolayers with 0.2% (w/v) INT [2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride in phosphate buffered saline: 140 mM-NaCl, 27 mM-KCl, 8 mM-Na2HPO4, 1.5 mM-KH2PO4, pH 7.3] or with neutral red [0.1% (w/v) in phosphate buffered saline] for 2 h at 27 °C (Fig. 1). These were counted and it was found that their number was proportional to the relative virus concentration (Fig. 2). Thus, each plaque was initiated by 1 plaque forming unit (p.f.u.; Davis *et al.* 1973). Plaques were also observed with overlay containing 1% agarose (Miles Laboratories, Inc.). It was observed that plaques were visualized equally well after staining with neutral red or with INT and that the plaque counts after staining correlated with the plaque number determined using the microscope.

The TCID50 titre of a virus preparation was determined by end point titration in TN-368 cells (Brown & Faulkner, 1975) and the ratio p.f.u.:TCID50 was found to be 0.05, whereas if the TCID50 determination was done with *S. frugiperda* cells, the p.f.u.:TCID50 value was 0.2. In another plaque assay experiment, the p.f.u.:TCID50 ratios were 0.17 and 0.6 based on TCID50 titrations with *T. ni* and *S. frugiperda* cells respectively. All plaque assays were done with *S. frugiperda* cells.

It appears from the TCID50 titration data that *S. frugiperda* cells are less sensitive as indicators of infection with *T. ni* MP3 (SF) than are *T. ni* cells. To investigate this phenomenon further, the efficiency of plating of the virus was determined in both *T. ni* and *S. frugiperda* cells. Microtest plates (Falcon Plastics) were seeded with either 2500 *S. frugiperda* cells per well in a 5 μl vol. or with 2000 *T. ni* cells per well. A 5 μl sample of *T. ni* MP3 (SF) containing 1.8 infectious units (IU), determined by a TCID50 titration using *T. ni* cells,
was added to each well. The plates were scored daily from 3 to 11 days p.i. and those wells showing a virus-induced c.p.e. were recorded. Wells containing \textit{T. ni} cells could be scored as long as 7 days p.i. and those with \textit{S. frugiperda} cells could be scored up to 11 days p.i. before the cells disintegrated. When plates were scored 3 days p.i., c.p.e. was observed in 27\% of wells seeded with \textit{T. ni} cells and 3\% of wells containing \textit{S. frugiperda} cells. The percentage of wells showing c.p.e. increased to 83\% for wells containing \textit{T. ni} cells, scored 7 days p.i., and 33\% for wells containing \textit{S. frugiperda} cells, scored 11 days p.i. Thus, \textit{S. frugiperda} cells are less sensitive as indicators of infection with \textit{T. ni MP3} (SF) than are TN-368 cells.

Other viruses which gave a linear dose response in the plaque assay on \textit{S. frugiperda} cells were the plaque-purified FP strain of \textit{T. ni NPV} used at the 3rd passage level in cell culture and the MP strain of \textit{Autographa californica NPV}, also at the 3rd passage level in cell culture. The MP and FP strains have been described previously (Potter, Faulkner & MacKinnon, 1976). MP and FP plaques could not be distinguished macroscopically.

TN-368 cells were also tested as indicator cells for the plaque assay of NPV using plates seeded with $5 \times 10^5$ to $10^6$ viable cells and \textit{T. ni MP3} (SF) as the inoculum. Microscopic examination of the plates 4 days p.i. showed that several foci of infection were present, comparable in size to those in \textit{S. frugiperda} cell monolayers. The monolayers were stained with INT, crystal violet or neutral red. In each case, the monolayer stained evenly and no plaques were visualized. Plaques observed in \textit{S. frugiperda} monolayers are areas of low cell density in a background of confluent cells. In \textit{T. ni} cell monolayers loci of infected cells form but these loci are not visible macroscopically as plaques, possibly because there is no major change in cell density at the foci.

If each infectious unit of virus added to the cells initiated the formation of a plaque, the relationship, $1 \text{TCID}_{50} \equiv 0.7 \text{p.f.u.}$ would hold (Davis \textit{et al.} 1973). Since $1 \text{TCID}_{50}$ was less than $0.7 \text{p.f.u.}$ in our experiments, the TCID$_{50}$ titration was a more sensitive assay than the plaque method. However, the plaque technique provides a simple means of cloning the virus and therefore will be valuable in genetic studies of these viruses.

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