Comparison of Microtiter Procedures with the Plaque Technique for Assay of Vesicular Stomatitis Virus

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This paper describes a microprocedure for the tissue culture assay of vesicular stomatitis virus (VSV) and compares the sensitivity of the method with the conventional plaque assay of viral concentration. Microtiter and plaque assay methods were used in titrations, neutralization tests, and thermonactivation studies with this virus in chick embryo fibroblasts (CEF) and L, HeLa, and PK cell lines. Titrations experiments with VSV by the microtiter procedure on preformed monolayers were significantly more sensitive than the plaque assay ($P = 0.05$). The results of the neutralization tests and thermonactivation studies also showed greater ability to detect residual virus by the microtiter procedure ($P = 0.10$). In addition, the microtiter procedure was simpler, less costly, and more rapid than the plaque assay.

The standard method for the determination of viral concentration by plaque count requires large quantities of costly reagents and considerable time. Consequently, micromethods for tissue culture were developed that proved to be accurate, more economical, and less time consuming than the macromethods. Microtiterations are based upon observation of the extent of cytopathic effect produced by a virus. Such procedures have evolved, in part, from the work of Takatsy (9), who introduced calibrated spiral loops and pipettes to perform serial dilutions in the wells of specially designed plates. Many of the components of the system of Takatsy were modified by Sever (8) and, more recently, by Rosenbaum et al. (7). The latter showed a close agreement between virus titers determined by the micromethod and those derived by the conventional tissue culture technique.

We were interested in determining whether microprocedures described by Rosenbaum and co-workers could be used with vesicular stomatitis virus (VSV). In addition, it was desirable to compare the sensitivity of the micromethod under different experimental conditions with the conventional plaque assay.

MATERIALS AND METHODS

Microtiter plastic plates and micropipettes were obtained from commercial sources (Linbro Chemical Co., Inc., New Haven, Conn.; Cooke Engineering Co., Alexandria, Va.). Two different types of microplates were employed in developing tissue culture procedures: one contained 48 flat-bottom wells (6 by 8 rows), each with a working capacity of 0.5 ml; the second contained 96 round-bottom cups (8 by 12 rows), each with a working capacity of 0.2 ml. Microplates were pretreated by soaking in 95% ethanol for 1 hr followed by several rinses with distilled water.

The micropipettes were made of semitransparent polypropylene, equipped with a stainless-steel dropper tube calibrated to deliver 0.025 ml per drop. A modification of the commercial pipette was developed which was considerably less expensive. These pipettes were easily assembled from a bevel-free 17-gauge needle, an observation tube, and a rubber bulb and provided uniform delivery (37 drops per ml). However, in the data presented in this report, the commercially available micropipettes were employed. These pipettes were rinsed with distilled water and autoclaved before use.

Preparation of chick embryo fibroblast monolayers. Monolayers of chick embryo fibroblasts (CEF) were prepared by the method of Dulbecco and Vogt (2) as modified by McClain and Hackett (5). Nine-to 11-day-old chick embryos were harvested, transferred to a sterile beaker, and washed three times with Dul-
becco's buffered saline. The embryos, stripped of head and appendages, were washed several times with saline and passed through a disposable syringe (without needle) into a trypsinization flask. The fragmented tissue was washed again with Dulbecco's buffered saline and allowed to settle to the bottom of the flask each time before the supernatant fluid was discarded. An appropriate volume of saline A containing 0.05% trypsin was added to the flask, and the suspension was agitated on a magnetic stirrer at room temperature for 30 min. After trypsinization, the larger tissue fragments were allowed to settle to the bottom of the flask, and the supernatant fluid was filtered through eight thicknesses of sterile gauze into a beaker. This fluid was transferred to graduated centrifuge tubes and centrifuged at 1,500 rev/min for 5 min in an International PR-1 centrifuge. The supernatant fluid was decanted, and the volume of packed cells was determined. A 1-ml amount of lamb serum was added to the packed cells to stop the trypsinizing action, and a 1:200 suspension (2 × 10^6 to 3 × 10^6 cells/ml) of the chick cells was made in lactalbumin hydrolysate in Earle's salt solution (LE) supplemented with 5% lamb (LE + S). The cell suspension was dispensed in 8-ml samples into 3-oz (90 ml) prescription bottles by using a Cornwall syringe, whereas calibrated micropipettes were used for the microtiter system.

Preformed monolayers. CEF monolayers were prepared in flat-bottom microplates by placing three drops (0.075 ml) of LE + S in each well of a microplate. This was followed by the addition of 1.5 × 10^6 to 2.0 × 10^6 cells per well contained in three drops of LE + S. The plates were rotated gently to insure mixing. Each well was overlaid with 0.075 ml of sterile mineral oil and incubated at 37 C for 24 to 36 hr. All media used contained penicillin (100 units/ml), streptomycin (0.5 mg/ml), and neomycin (0.1 mg/ml).

Before titration, the mineral oil and growth medium were discarded and the monolayers were washed twice with Dulbecco's buffered saline. When ready for titration, two drops of LE were delivered to each well which was to be infected with virus, whereas wells designated as controls received three drops of LE. One drop of virus dilutions, previously prepared by 10-fold increment dilutions in test tubes with LE as diluent, was then delivered to six replicate wells. The plates were then overlaid with mineral oil and reincubated for 48 hr at 37 C.

At 48 hr postinoculation, monolayers in microplate wells were observed for the presence of cytopathic effect (CPE). This was shown by cellular degeneration and by the "rounding up" of infected cells. CPE was scored on the conventional basis of 0 through 4+.

Zero indicated a normal confluent monolayer; 1+ represented a visual estimation of 25% CPE; 2+, 3+, and 4+ represented 50, 75, and 100% CPE, respectively. The percentage of CPE was determined by visual examination of the entire monolayer, contrasting the number of infected cells to normal cells.

Infectivity titer was expressed as the highest dilution of virus which infected the monolayers in 50% of the wells. The titer, designated as ID_{50}, was computed by the procedure described by Reed and Muench (6).

The ID_{90} titer reported was computed from the average count of two microplates used per dilution. A graded cytopathic response of 1+ to 4+ was observed during the first 12 to 36 hr after incubation of infected microplates. An all-or-none response was present at the end of the assay period at 48 hr, since monolayers infected with virus always showed 4+ CPE and uninfected monolayers showed no evidence of cellular degeneration.

A similar procedure was followed to prepare CEF monolayers in round-bottom microplates. Since these plates contained more wells, they allowed a greater number of assays to be carried out as well as occupied less incubation space. Two drops of LE + S were added to each well, followed by the addition of one drop of CEF cell suspension (2 × 10^6 to 3 × 10^6 cells/ml). The wells were overlaid with 0.05 ml of mineral oil and incubated at 37 C for 24 to 36 hr. Washing and titration procedures were the same as those described previously.

For production of other monolayers in round-bottom microplates, 7,000 to 8,000 L cells or 2,000 to 4,000 HeLa cells suspended in 0.075 ml of Eagle's minimum essential medium (MEM) with 10% fetal bovine serum were delivered to each well. Porcine kidney (PK) monolayers were also prepared by suspending 4,000 to 6,000 PK cells in 0.075 ml of LE + S.

The wells were overlaid with mineral oil and incubated at 37 C until monolayers were confluent at 72 hr. Washing and titration procedures were described previously.

Suspended cell preparations. Similar concentrations of CEF or line cells were used for experiments with suspended cells. In this case, virus dilutions were added to each plate, followed by addition of cell suspensions in the appropriate growth medium. The main advantage of this technique was that the cells and virus were added at the same time, thus eliminating the time required for production of monolayers as well as washing and titration procedures.

Plaque assay. Infectivity titers were determined by the plaque assay method (1). Cell monolayers in 3-oz prescription bottles, washed twice with 5-ml volumes of Dulbecco's buffered saline, were inoculated by using five bottles per dilution with 0.1-ml samples of the virus dilution.

After 1 hr of adsorption at room temperature, CEF monolayers were overlaid with 8 ml of nutrient agar medium containing neutral red. L, HeLa, and PK monolayers were each initially overlaid with 8 ml of nutrient agar medium without neutral red; a second overlay incorporating neutral red was added after 48 hr to these monolayers. Plaques appeared as clear, unstained areas against a reddish background after 72-hr incubation titers were expressed as plaque-forming units (PFU) per milliliter of undiluted viral suspension.

Virus production and concentration. The Odgen strain of the New Jersey serotype of VSV was grown in 24- to 30-hr CEF monolayers in 16-oz (450-ml) prescription bottles by the procedure described by McClain and Hackett (5). Virus suspensions were harvested after 12 to 14 hr and clarified by centrifuga-
tion at 1,000 × g for 10 min; the virus was concentrated by subsequent centrifugation at 17,000 × g for 90 min. The viral pellets were pooled and resuspended to a 10-fold concentration and stored at −60 C.

Neutralization tests. Anti-VSV stock serum (provided by R. St. John) was employed to assay various concentrations of VSV. The serum was heat-inactivated at 56 C for 30 min before use to destroy labile substances that have antiviral activity or affect neutralization. Serial 10-fold dilutions of virus and serum prepared separately in test tubes were made with LE as diluent. A sample (1.0 ml) of each serum dilution was then mixed with decreasing virus dilutions (1.0 ml) in test tubes, and the mixture was incubated at room temperature for 30 min. The concentration of the remaining unneutralized virus was then determined by comparative titrations by using the microtiter and plaque methods previously described. Monolayers in microplate wells were inoculated with 0.025-ml amounts of the same serum-virus mixtures used to inoculate 3-oz bottles. After 48 hr of incubation, ID₅₀ titers were calculated and were based on the degree to which the serum inhibited CPE. This inhibition of CPE was compared to the degree by which the serum reduced plaque counts.

Thermoinactivation study. Virus samples were diluted 10-fold in MEM in French square bottles, pre-tempered to 34 C for 1 hr in a water bath. The inactivation time course was begun when the virus sample was added to the MEM. Heated samples were taken at various time intervals and immediately cooled in an ice bath. All subsequent dilutions were made in LE. These dilutions were used to inoculate CEF monolayers in microplate wells and in 3-oz prescription bottles. CPE was determined by microscopic observation, and infectivity titers (ID₅₀) were calculated by the method of Reed-Muench (6) as well as by plaque counts.

Statistical analyses. A two-way analysis of variance was performed to determine whether there was a significant difference between the methods of viral assay (ID₅₀ titers from suspended cell preparations, preformed monolayers, and plaque counts) and cell lines (CEF, L, HeLa, and PK) and also to determine if interaction was present. In addition, Duncan's new multiple range test (3) was carried out to determine individual differences between methods and cell lines.

RESULTS AND DISCUSSION

VSV titrations were compared by using microtiter suspended cell and preformed cell monolayer methods as well as the routine plaque assay. The results shown in Table 1 indicate that the microtiter procedure, with preformed monolayers, produced higher infectivity titers than assays made with either cell suspensions or plaque formation. A two-way analysis of variance showed that these differences were statistically significant (P = 0.05). No statistically significant difference in sensitivity was revealed between the cell suspensions and the plaque assay method. However, the microtiter procedure facilitated a more rapid assay of VSV with no loss in accuracy. The presence of lamb serum throughout the incubation of

Table 1. Comparative titrations of vesicular stomatitis virus by microtiter and plaque methods with suspended cells and preformed monolayers

| Expt no. | Cell line | Suspended cells | Preformed monolayers | Plaque assay (PFU/ml) |
|----------|-----------|-----------------|----------------------|----------------------|
| 1        | CEF       | 5.5 × 10⁸       | 8.7 × 10⁸            | 1.2 × 10⁸            |
|          | L         | 2.2 × 10⁸       | 1.7 × 10⁸            | 3.6 × 10⁸            |
|          | CEF       | 1.0 × 10⁹       | 1.4 × 10⁹            | 2.5 × 10⁹            |
| 2        | HeLa      | 8.6 × 10⁴       | —                    | 1.8 × 10⁸            |
|          | PK        | 1.0 × 10⁶       | —                    | 1.6 × 10⁹            |
|          | CEF       | 9.8 × 10⁸       | 3.0 × 10⁹            | 3.5 × 10⁹            |
| 3        | PK        | —               | 8.5 × 10⁸            | 5.2 × 10⁸            |
|          | CEF       | —               | 1.3 × 10⁹            | 3.7 × 10⁹            |
| 4        | HeLa      | 1.4 × 10⁶       | 3.4 × 10⁸            | 6.2 × 10⁹            |
|          | CEF       | 6.3 × 10⁶       | 8.6 × 10⁸            | 2.0 × 10⁹            |
|          | L         | 1.5 × 10⁸       | 4.1 × 10⁸            | 3.0 × 10⁹            |
| 5        | PK        | 8.2 × 10⁸       | 1.5 × 10⁷            | 7.0 × 10⁷            |
|          | HeLa      | 1.7 × 10⁹       | 3.5 × 10⁷            | 4.8 × 10⁷            |

* Microtiter ID₅₀ (average of two microplates).
* Not done.

Fig. 1. Survival curve of vesicular stomatitis virus stored at 4 C as measured by microtiter and plaque assay methods on chick embryo fibroblast monolayers.
microtiter suspended cell plates did not affect virus titers, since neutralizing antibodies to VSV were not observed.

The reproducibility of the microtiter system was previously investigated in work presented elsewhere (7, 8; L. J. Rosenthal, M. A. Thesis, Southern Illinois Univ., 1967). In our laboratory, with ID\textsubscript{50} titers obtained from a series of five microplates with cell suspensions and preformed monolayers, the standard error of the mean was computed to be 0.48 and 1.10, respectively. In the data presented in Table 1, standard errors were not determined since the ID\textsubscript{50} given represented the average ID\textsubscript{50} from two microplates.

In other experiments presented in Fig. 1, with a sample of VSV stored at 4°C, titrations of residual infectivity were carried out periodically for various times as long as 100 days by microtiter and plaque methods on preformed CEF monolayers. The ID\textsubscript{50} titers were calculated from two replicate microplates with six wells per dilution, whereas plaque titers were determined from the average count of four bottles used per dilution. Regardless of the storage time, the ID\textsubscript{50} titers were statistically higher ($P = 0.05$) than the plaque counts. Both methods showed a close agreement in the rate of inactivation of VSV stored at 4°C.

Comparative virus neutralization tests were carried out by using both microtiter and plaque methods on CEF monolayers. The microtiter procedure was shown statistically ($P = 0.10$) to be more sensitive in detecting unneutralized virus than the plaque procedure. These data are presented in Fig. 2, and the individual experiments are designated with the capital letter A, B, and C. In these experiments, the ID\textsubscript{50} titers paralleled plaque neutralization results and revealed similar viral neutralization characteristics.

An evaluation of thermoinactivation data demonstrated again that the microtiter procedure with preformed cell monolayers was statistically ($P = 0.10$) a more sensitive measure of viral infectivity than plaque formation. The results of an inactivation experiment with VSV are presented in Fig. 3. In all samples taken during inactivation, the ID\textsubscript{50} titers were higher than plaque counts. There was, however, good agreement in the rate of inactivation of VSV determined by either the microplate or plaque methods. Thus, the microtiter procedure was applicable for inactivation studies of VSV and was simpler to do, less costly, and as accurate as the plaque assay.

We conclude that the micromethod either with
cell suspensions or preformed monolayers was found to be useful in titration, neutralization, and inactivation experiments with VSV. In addition, the micromethod compared favorably with the plaque method for accuracy, with the advantage of more economy and less effort.

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