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Reproducible Plaquing System for Rabies, Lymphocytic Choriomeningitis, and Other Ribonucleic Acid Viruses in BHK-21 / 13S Agarose Suspensions

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BHK21/13S-agarose suspension cultures provide an excellent plaquing system for rabies and lymphocytic choriomeningitis viruses. The system has facilitated accurate and reproducible assay of serum neutralization and cloning of these viruses. Rubella, reo, and Kern Canyon viruses may also be assayed by this technique.

We report here a technique for plaque assay in BHK-21 cell cultures (5), which has been successfully used in our laboratory for titration, cloning, and serum neutralization of rabies virus and lymphocytic choriomeningitis (LCM) virus. This assay system has also proved effective for assay of other ribonucleic acid (RNA) viruses that have been difficult to plaque, such as rubella and reoviruses.

Although plaque formation of rabies in chick fibroblasts has been reported (9), this technique has not provided a consistent assay tool in most laboratories. The capacity of BHK-21 embryonic hamster cells to support the growth of rabies virus in monolayer cultures was described previously (3).

MATERIALS AND METHODS

The Pitman-Moore strain of fixed rabies virus derived from rabbit brain was used in these studies after 52 passages in WI-38 cells (7) and two additional passages in Nil-2 cells of hamster origin (2). The cells used in these experiments were a subline of BHK-21 clone 13S, adapted to suspension cultures (6).

After observing that uninfected BHK-21/13S cells could be maintained in the presence of agarose for 1.5 to 2 weeks, we attempted to produce rabies virus plaques by use of a technique similar to that described by P. D. Cooper (1). Cells were propagated in 1-liter Blake bottles, in BHK-21 medium (5), supplemented with 10% inactivated fetal calf serum. An inoculum of 15×10^6 cells in 5 to 6 days yielded from 100×10^6 to 200×10^6 cells per Blake bottle.

Cells suspended in the medium were centrifuged, pooled with cells removed from the surface of the bottle by 0.25% trypsin, and resuspended to a concentration of 8×10^6 to 10×10^6 cells/ml in BHK-21 medium containing 2% fetal calf serum. Dilutions of virus prepared in the same medium were added to the cells in a ratio of 1 volume of virus to 9 volumes of cell suspension. The virus-cell mixture was agitated repeatedly for 1 hr at 35 C.

After adsorption of virus, the cell-virus suspension was plated as follows: 4 ml of MS medium without bovine albumin (4) containing 2% inactivated calf serum and 0.4% melted agarose (Seakem; Bausch and Lomb, Rochester, N.Y.) was allowed to harden as an underlayer in a 60-mm plastic Falcon petri dish. The overlayer was prepared by mixing the incubated virus-cell suspension in equal volume with the above MS medium. One ml of this suspension was then pipetted onto the agarose underlayer and spread evenly by rotating the petri dish. Each dish thus contained a final concentration of about 5×10^6 cells in a thin layer.

The cultures were placed in a humidifier incubator in an atmosphere of about 4% CO_2 in air. Plaques were made visible by adding 3 ml of the 0.4% agarose overlay containing 10^{-4} g of neutral red/ml on the day of reading.

RESULTS AND DISCUSSION

Rabies virus plates were prepared as described in Materials and Methods. After 6 to 7 days of incubation, an average plaque size of about 3 mm was achieved. Typical rabies plaques are illustrated in Fig. 1.

The following experiments proved the specificity of the rabies plaques: (i) no plaques appeared after prior incubation of virus suspensions

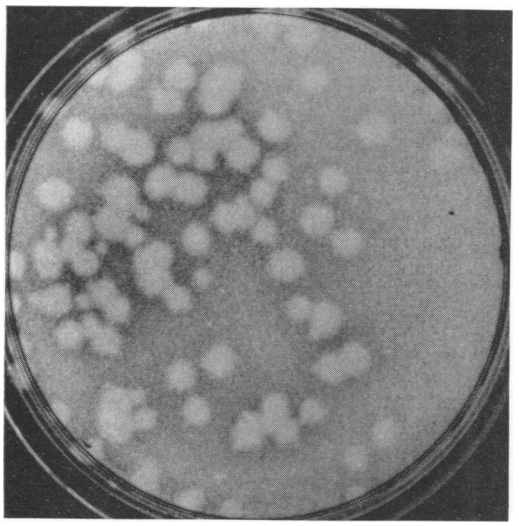

FIG. 1. *Typical rabies plaques developed by neutral red staining at 7 days postinfection.*

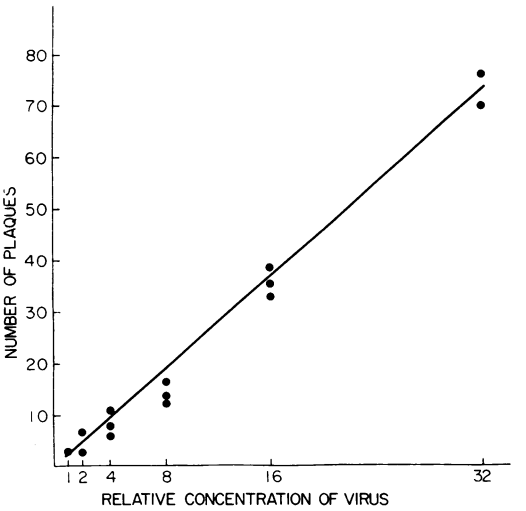

FIG. 2. *Linear function between the number of plaques per individual plate and rabies virus dilution.*

with specific anti-rabies serum; (ii) when a virus clone was isolated from a plaque and used for infection of new BHK-21, Nil-2, or WI-38 cells, specific rabies antigen was detected in the cells by staining with fluorescent antibodies; (iii) after three serial plaque passages, identity of rabies virus was confirmed by a serum neutralization test in mice. In further confirmation of the above experiments, plaque areas were sectioned for electron microscopy by K. Hummeler of Childrens' Hospital of Philadelphia, and were found to contain typical rabies virus particles.

The utility of this plaque assay system for quantitation of infectious rabies is shown in Fig. 2. The figure shows that plaque number was linear with respect to dilution. A good correlation was also obtained between a standard World Health Organization serum neutralization test in mice and an in vitro test by plaque reduction with three different anti-rabies sera of human, equine, and rabbit origin.

LCM virus was plaqued by a technique identical to that just described for rabies virus. Plaques were obtained with three strains of LCM virus isolated in our laboratory (8). Plaques that were similar in size and appearance to the 7-day rabies plaques were already fully developed after only 4 days of incubation (Fig. 1). When clones of LCM virus were isolated and used for infection of new cultures, the presence of specific viral antigen could be detected in WI-38, BHK-21 and Nil-2 cells after staining with fluorescent anti-LCM globulins.

The BHK-21/13S agarose suspension technique is now the preferred method in our laboratory for the routine plaque assay of BHK-21/13S-adapted rubella virus strains. Rubella plaques may be read at 6 days after infection and, at this time, are from 3 to 4 mm in diameter. A serum neutralization test with paired acute and convalescent antisera from an experimentally infected rubella patient during acute and convalescent stages has proved the specificity of these plaques.

Reovirus III, Dearing strain, obtained from C. Gauntt of this Institute, was also plaqued successfully in this agarose suspension system. Plaques 1 mm in diameter were obtained in 6 days. James Campbell, also of this Institute, has recently successfully tested this system with Kern Canyon virus.

In addition, an accompanying paper (10) describes the use of this method for the growth of mycoplasma contaminants of virus pools which formed easily visible colonies. This fact enabled us to derive pure rabies and LCM clones from two virus stocks which had become contaminated with mycoplasma (10).

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