NOTES

Plaque Assay of Rabies Virus on Porcine Kidney Cell Monolayers

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The ERA strain of rabies virus was adapted to growth on monolayers of a porcine kidney cell line (PK-2A). A reproducible plaque assay system was subsequently developed, which appears to be satisfactory for conducting plaque neutralization tests.

Primary cell cultures and established cell lines have been used for the propagation of rabies virus with varying degrees of success. Exposure of mammalian cells in tissue culture to rabies virus results in what has been termed a carrier type of infection (8). No cytopathic effect is usually observed, and infected cultures often can be maintained through many cell transfers.

Several rabies plaque assay tests have been reported (3, 5, 6, 9); in the United States, however, only the method of Sedwick and Wiktor (6), using BKH-21/13S cells in agarose suspension has been used to any appreciable extent as a laboratory tool.

We describe here the adaptation of the ERA strain of rabies virus to growth on the PK-2A cell line, with subsequent development of a reproducible plaque assay system (1).

PK-2A cells were maintained in 32-ounce (ca. 946 ml) prescription bottles. Medium consisted of Eagle minimal essential medium, supplemented with L-glutamine, nonessential amino acids, and 10% fetal calf serum.

The ERA strain of rabies virus was carried through 14 serial passages in chicken embryo fibroblasts and then 164 passages in PK-2A cells. For each passage, undiluted infective fluid was transferred to previously prepared confluent monolayers. Cytopathic effect was first noticed after five passages in PK-2A cells and became progressively more prominent as the passage level increased; however, when tested at passage level 92, plaques were not easily or consistently visible.

For the plaque procedure, 8 ml of cell suspension (5×10^5 cells/ml) was added to each 60-mm well of a four-well plastic plate (Linbro

Chemical Co., Inc., New Haven, Conn.). The plates were placed in a humidified 35 C incubator in an atmosphere of 3% CO₂ in air and incubated until monolayers were confluent. Growth medium was removed and 0.2 ml of diluted virus was placed in the center of each well. After adsorption for 1 h at 35 C, excess inoculum was removed by aspiration and overlay was added.

A semisolid overlay was prepared by mixing 2× Eagle minimal essential medium with an equal amount of 1.5% carboxymethylcellulose (Hercules, Wilmington, Del.). This was supplemented with 5% fetal calf serum. Eight milliliters was added to each 60-mm well. Infected cultures were incubated at 35 C undisturbed for 5 days. The semisolid overlay was aspirated and 3 ml of 0.05% neutral red was added for staining. Plaques were also clearly visible without staining.

Initially, plaques were irregular in size and clarity; most were barely visible. In an attempt to select for virus that produced clear plaques, overlay medium located immediately over several plaques that were most clearly visible was carefully removed with a Pasteur pipette, diluted 10^{-3} through 10^{-6} , and passed to new plaque plates. Selection and passage of individual clear plaques was repeated serially three additional times until a homogeneous viral population that produced clear plaques was obtained (Fig. 1).

The plaque assay procedure was tried using the virus described and other types of tissue culture, including BHK-21 cells, Vero cells, primary dog kidney cells, and a related porcine kidney cell line (PK-15). Plaques were not formed on these cells in any instance.

FIG. 1. Plaque titration of rabies virus on PK-2A monolayers. (A) 10^{-3} ; (B) 10^{-4} ; (C) 10^{-5} ; (D) 10^{-6} .

Several variations in the procedure did not affect the ease with which plaques were obtained. These variations included the use of agar or tragacanth overlay instead of carboxymethylcellulose and the addition or deletion of polyions (4). Using the plaque technique as described, reproducible results were obtained on 25 tests conducted over a period of 6 months.

Specificity of the plaques was determined by neutralization with four different antisera: the National Institutes of Health reference anti-rabies serum; an anti-rabies serum of burro origin supplied by Center for Disease Control; human serum obtained from individuals vaccinated with duck embryo vaccine; and canine serum obtained from dogs after vaccination with inactivated CVS rabies vaccine. Serial two-fold serum dilutions were made, mixed with an equal amount of virus diluted to contain 50 to 100 plaque-forming units per 0.1 ml, and the mixture was incubated for 60 min at 37 C. Two-tenths milliliter of virus-serum mixture was added to each well and allowed to adsorb for 60 min at 35 C. Monolayers were then washed with tissue culture medium, overlayed, and incubated for 5 days as described above. The semi-solid overlay was removed by aspiration and 3 ml of 0.05% neutral red was added for staining. Plaque neutralization end points were recorded as the highest dilution of serum causing 50% or greater reduction in plaque count. The

TABLE 1. Comparison of antibody titers to rabies virus obtained by plaque neutralization, rapid fluorescent focus inhibition test (RFFIT), and mouse neutralization

Serum	Antibody titer ^a		
	Plaque Neutralization	RFFIT	Mouse Neutralization
NIH reference ^b	1:256	1:232	1:130
Burro	1:256,000	1:187,000	1:115,000
Human			
Nonimmunized	<1:2	<1:5	<1:3
Immunized	1:64	1:56	1:33
Dog			
Nonimmunized	<1:2	<1:5	<1:3
Immunized	1:8	1:11	1:4

^a All titers given as the average of 2 tests.

^b NIH, National Institutes of Health.

same sera were tested by the World Health Organization neutralization test in mice (2) and the rapid fluorescent focus inhibition test (7).

The results of the neutralization tests (Table 1) confirmed the specificity of the plaques and indicated that the system has good potential for use in measuring rabies neutralizing antibody.

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