Plaque Assay of Japanese B Encephalitis Virus on Hamster Kidney Monolayers.* (27368)

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The usefulness of hamster kidney cell (HKC) cultures for propagation and detection of Japanese B encephalitis (JBE) virus has been well established(1,2,3). It has been reported that JBE virus forms plaques in chick embryo(4,5,6) and duck kidney cell cultures(7) under agar overlay. However, plaque formation by JBE virus in monolayer cultures of HKC has not been successful(2, 6). This report describes plaque formation with JBE virus in monolayer cultures of HKC, using double agar overlay(8). It was found that JBE virus could produce distinct plaques with clear centers and sharp boundaries.

Materials and methods. The JBE virus used was the Nakayama strain which had been through 46 passages in mouse and 27 passages in HKC cultures(2,3). JBE virus antiserum was obtained from guinea pigs inoculated 3 times at 10-day intervals with JBE virus in its 48th mouse brain passage. Primary cell cultures of HKC and chick embryo were prepared in 3 oz. bottles. These were nourished in medium consisting of 0.5% lactalbumin hydrolysate in Hanks' balanced salt solution, 4% calf serum and antibiotics (9,2). Phosphate-buffered saline (PBS) (pH 7.0) was used for washing cultures(10). Two-tenths per cent bovine albumin in 0.5% lactalbumin hydrolysate medium served as diluent for titration of virus. For the first agar overlay the medium was made by mixing equal parts of 3.0% agar in sterile distilled water and the following: 2.0% lactalbumin hydrolysate in Hanks' balanced salt solution (4× concentrated, without either phenol red or $NaHCO_3$) 25 ml; calf serum (inactivated at 56°C for 30 minutes) 4 ml (or bovine albumin, 2.0% stock solution, 4 ml); $NaHCO_3$ (4.0%) 3 ml; antibiotics (200 units of penicillin, 40 mg of streptomycin, 10 units of polymyxin/ml) 1 ml; sterile distilled water 17 ml. The second overlay was similar to the first agar overlay medium except that it contained neutral red at 1:30,000.

A. *Plaque assay.* After removal of the medium, monolayers were inoculated with 0.1 ml of appropriate dilutions of virus. These were incubated at 37°C with occasional rocking to prevent drying of cells and to improve distribution of the virus. After 1 hour, 6 ml of first agar overlay were added. After the agar solidified, the bottles were turned over and placed at 37°C. On the fourth day of incubation, a second overlay of 6 ml was added, incorporating neutral red.

B. *Neutralization tests* were performed by mixing 0.5 ml of virus containing an estimated 100 PFU/0.1 ml with 0.5 ml of serum in appropriate serial 2-fold dilutions. The mixture was incubated at 37°C for 2 hours, 0.2 ml of the mixture was inoculated per bottle and the double overlay was added as described above. A control titration using serial virus dilution with normal control serum at a 1:10 dilution was carried out at the same time. Final readings were made on the sixth day of inoculation. Comparative titers by the CPE (cytopathic effect) method, using 100 TCD_{50} were performed in tubes simultaneously by the method as described above.

C. *Relationship of virus dilution to plaque number.* Starting at a 10^{-5} dilution of virus stock, 0.1 ml of serial 2-fold virus dilutions were inoculated into each bottle. After 1 hour, bottles were removed from the incubator, washed with PBS twice and overlaid with agar medium as described above.

Results and discussion. A. Fig. 1 shows the plaques produced by JBE virus at 10-fold dilutions in HKC bottle cultures, 6 days after

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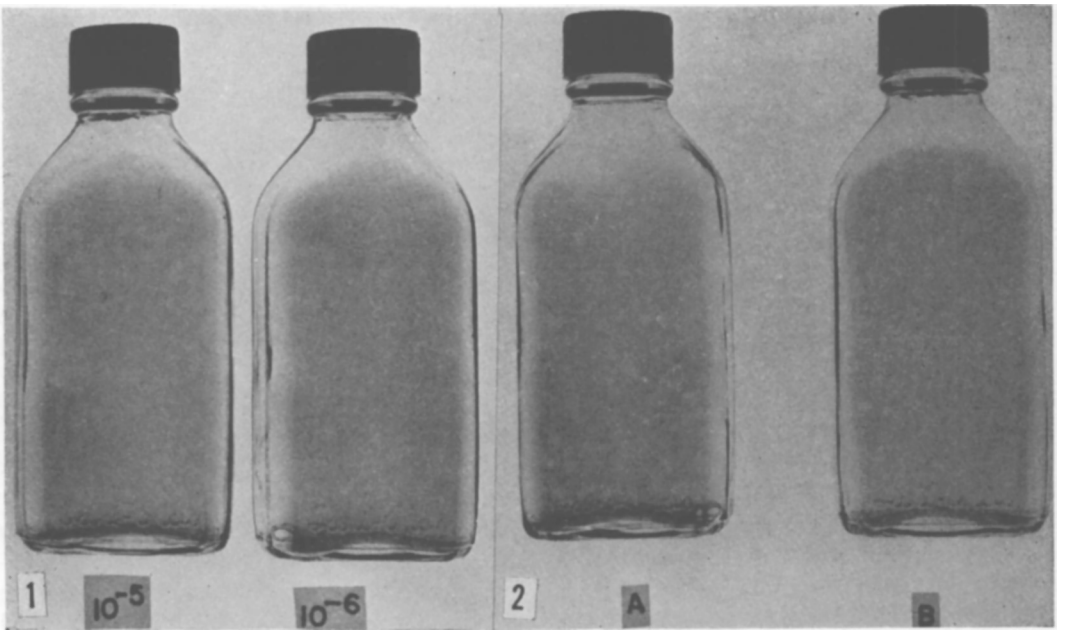

FIG. 1. Quantitative titration of JBE virus, Nakayama strain (27th passage in hamster kidney cultures) 6 days after seeding serial 10-fold dilutions, 0.1 ml/3 oz bottle.

FIG. 2. Plaque formation of a 10^{-5} dilution of JBE virus in hamster kidney culture (A) and chick embryo culture (B), 6 days after seeding.

seeding. Small but distinct plaques with sharp boundaries were obtained as early as 3-4 hours after overlay with neutral red included in the second agar overlay on the 4th day after seeding and they increased to 4-6 mm in diameter by the sixth day (Fig. 1). There was little increase in number after the sixth day. On the other hand, HKC bottle cultures which received a single overlay containing neutral red became decolorized between 48 and 72 hours after seeding as previously reported from this laboratory(2). Demonstration of plaque formation in HKC bottle cultures using a single agar overlay apparently was not possible, regardless of the time of adding the neutral red to the HKC cultures.

JBE virus plaques were also obtained in chick embryo bottle cultures by using the double overlay described above. As shown in Fig. 2B, the plaques were not as clear and the boundaries were not as distinct as those shown in HKC monolayers (Fig. 2A). They were tiny and hazy with less distinct margins and included a number of cells which retained the neutral red stain in the center of the plaques(6), which could not be observed in the plaques produced on HKC monolayers. The plaques on chick embryo increased to 1-3 mm in diameter.

B. Table I gives a typical result of JBE virus neutralization test. The virus was neutralized by specific JBE virus guinea pig antiserum. The 50% protection titer of guinea pig antiserum against 100 TCD_{50} was 1:900 when tested by the conventional tube method; however, about 80% plaque reduction was obtained at a dilution of 1:5,120 by the plaque technic.

C. The relationship between plaque count and virus concentration is shown in Fig. 3. A linear relationship was observed between number of plaques and virus concentration, indicating that each plaque was produced by a single virus particle(11). The isolation of virus clones from plaques would appear to be simple. However, Mosley and Enders(12) point out that additional overlays of the agar following virus multiplication increase the hazard of viral contamination from the glass and agar surface rendering selection of a pure clone less likely. Tests to determine this in

TABLE I. Comparison of Plaque and CPE Methods of Demonstrating Titers of Serum-Neutralizing Antibody to JBE (Nakayama Strain) Virus.

Titer of virus challenge	Antiserum dilution 1:320	1:640	1:1280	1:2560	1:5120	1:10,240	1:20,480
100 PFU ‡	0*	0	1	5	21	42	64
100 TCD_{50}‡	0/3†	1/3	2/3	3/3	3/3	3/3	3/3

* Avg PFU/bottle.
† No. of tube cultures showing CPE/No. inoculated at each dilution.
‡ Actual simultaneous virus titers, 90 PFU and 85 TCD_{50} respectively.

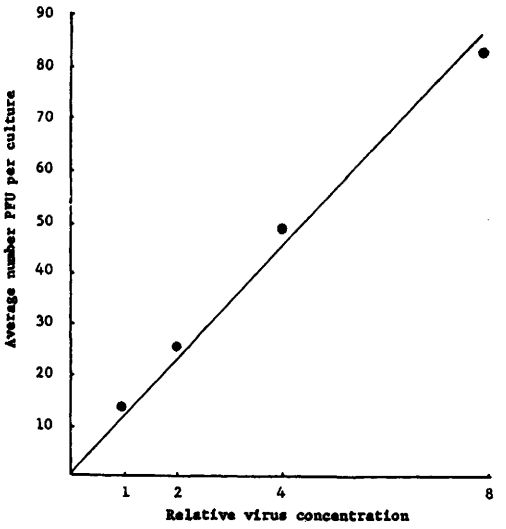

FIG. 3. Proportionality between number of plaques and virus concentration.

the system reported here were not carried out. Further quantitative studies with JBE virus by the plaque method will be published later.

Summary. (1) Using a double agar overlay with neutral red in the second overlay only, JBE virus produced distinct plaques with sharp boundaries in monolayer cultures of HKC. They were clear and 4-6 mm in diameter after 6 days of cultivation. In contrast, the plaques produced in the same manner by JBE virus on chick embryo bottle cultures were hazy and 1-3 mm in diameter. (2) Plaque reduction neutralization tests were readily performed and proved to be more sensitive than these performed with the CPE method using monolayers in tubes with fluid medium. (3) A linear relationship was observed between plaque counts and virus concentration, permitting precise quantitative virus assay.

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