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In Vitro Studies with Modoc Virus in Vero Cells: Plaque Assay and Kinetics of Growth, Neutralization, and Thermal Inactivation

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A sensitive and quantitative assay system is described for plaquing Modoc virus in Vero cells. Neutralizing antibodies to Modoc virus could be detected by using this in vitro system by their interference with viral plaque formation. Virus was readily neutralized within 30 min at 37 C by a 1:10 dilution of hyperimmune hamster serum. The rate of neutralization and the total amount of virus neutralized was not altered significantly by the addition of 20 U of guinea pig complement to the hyperimmune hamster serum. A study of the growth of Modoc virus in Vero cells is also presented. After an initial latent period of 20 h, viral titer increased exponentially for 20 h. By 83 h after infection, 8,000 plaque-forming units of virus were detected per cell. The stability of viral infectivity in phosphate-buffered saline at pH 7.4 was evaluated. No reduction in viral titer was detected after 3 days at 7 or 22 C. A continuous decrease in infectivity at 37 C was observed, however, throughout the observation period.

Modoc virus is classified taxonomically as a group B arbovirus on the basis of its antigenic relationship to viruses within this group, although there is no evidence that this virus is vectored by an arthropod (1, 3). All reported isolations of Modoc virus have been made from infected tissues of the deer mouse, *Peromyscus maniculatus* (3, 10). An epidemiological study in California of undiagnosed diseases associated with the central nervous system suggested that Modoc virus was responsible for one case of aseptic meningitis in a young boy (W. C. Reeves, unpublished data). This boy had reportedly played with a "sick mouse" at a cabin situated in the Sierra foothills several days before onset of disease. Johnson (4) reported that hamsters inoculated with Modoc virus became chronically infected and continued to shed the virus in their urine for several months after infection. These isolated observations indicated that Modoc virus might be transmitted horizontally via infected urine and create a potential health hazard for man. Studies were needed to determine whether Modoc virus produced a chronic infection in deer mice.

Before more extensive investigations of Modoc viral infections in experimental animals could be undertaken, it was deemed necessary to develop a system other than intracerebral (ic) inoculation of suckling mice (SM) for assay of Modoc virus. The prototype strain (M544) had been shown to produce cytopathogenic effects in the BHK-21 line of baby hamster cells (5) and to produce plaques in both African green monkey (Vero) and rhesus monkey kidney (LLC-MK2) cell cultures (9). This report describes the parameters necessary for a sensitive and quantitative plaque assay of Modoc virus in Vero cells. To further characterize Modoc virus, growth rate in Vero cells, neutralization kinetics, and thermal inactivation rate were determined.

(This is part of a dissertation submitted to the Univ. of California in partial fulfillment of the requirement for the Ph.D. degree.)

MATERIALS AND METHODS

Virus. The prototype strain (M544) of Modoc virus, which was isolated from the mammary gland of a lactating deer mouse (1), was obtained from Harald N. Johnson. Upon receipt the virus had undergone only one intramuscular (im) passage in hamsters, and the material received was viremic blood from these hamsters. The virus was passaged twice in SM by ic inoculation, and a stock virus was prepared as a 10% homogenate of infected mouse brain in 50% heat-inactivated (56 C for 30 min) fetal calf serum (FCaS) and 50% beef-heart infusion broth. After clarification by

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centrifugation at 400 g for 10 min, the supernatant fluid was ampouled and stored at −70 C.

Cell cultures. Vero cells (11) were obtained from the American Type Culture Collection. Stock cultures of Vero cells were grown in 16-ounce (approximately 473 ml) glass prescription bottles in nonautoclavable Eagle minimal essential medium (MEM) that was prepared in Earle balanced salt solution (BSS) and supplemented with 5% heat-inactivated FCaS. All media contained 1.6 g of $NaHCO_3$ per liter, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. The complete medium was sterilized by membrane filtration (Millipore membrane filters, Millipore Corp.). Cultures that were to be used for viral assay were grown in plastic petri dishes (60 by 15 mm) in the same medium except that autoclavable MEM was used. All cultures were incubated at 37 C in a humidified atmosphere of 5% CO_2 in air.

Viral assay. Modoc virus was assayed by plaquing in Vero cells. Serial 10-fold dilutions of virus were prepared in Dulbecco phosphate-buffered saline (PBS) that contained 0.75% bovine albumin fraction V (BA). Monolayer cultures were rinsed once or twice with 3 ml of PBS, and virus in 0.2-ml volumes was adsorbed onto each of two replicate cultures for 1.5 to 2 h at 37 C. After rinsing with PBS, each culture was overlayed with 5 to 6 ml of medium that consisted of 1.5% methyl cellulose (4,000 centipoises) in autoclavable MEM prepared in Earle BSS and supplemented with 2% heat-inactivated FCaS. Concentrations of $NaHCO_3$ and antibiotics were the same as for the growth medium. For visualization of plaques, each culture was overlayed after 4 days of incubation at 37 C with 6 ml of the same medium except that it contained neutral red (1:36,000) and the methyl cellulose was replaced by 1% purified agar. Plaques were counted on the third day after staining and titers were expressed in plaque-forming units (PFU).

In one experiment, virus was titrated simultaneously by plaquing in Vero cells and by ic injection of 1- to 2-day old SM (0.01 ml per mouse) in order to compare the sensitivities of the two assay methods. The mean lethal dose in SM was calculated by the method of Reed and Muench (8).

Preparation of immune hamster sera. The SM passaged stock of Modoc virus was inoculated ic into suckling Syrian hamsters. When the hamsters were moribund, the infected brains were removed and stored at −70 C. For immunization, 6- to 8-week-old hamsters were injected weekly for 7 weeks with a 20% infected hamster brain suspension in saline. The first injection was given im (0.05 ml) and subsequent inoculations were given intraperitoneally (1.0 ml). The virus suspension was emulsified in an equal volume of Freund incomplete adjuvant for the third and fifth injections. Hyperimmune serum was obtained 10 to 12 days after the last injection.

Plaque formation in Vero cells. Studies were done to determine the time necessary for optimal adsorption of Modoc virus onto Vero cells. Monolayer cultures were inoculated with approximately 60 PFU of virus that were contained in 0.2-ml volumes and the inoculated cultures were incubated at 37 C. At various intervals after inoculation, four plates were removed and rinsed three times with 5 ml of PBS to remove unadsorbed virus. The plates were then overlayed with medium as described previously.

The methyl cellulose technique was evaluated as a quantitative assay for Modoc virus on the basis of the plaquing of serial twofold dilutions of a suspension that contained approximately 100 PFU per 0.2 ml. Each dilution of the suspension was plaqued in seven cell cultures.

Viral growth. To study the growth of Modoc virus in Vero cells, monolayer cultures were infected at a multiplicity of infection of one to two. After adsorption of virus for 1.5 h at 37 C, 2.8 ml of growth medium was added to each culture. To determine the amount of free virus, 0.5-ml samples were removed from each of three infected cultures at various times after adsorption, pooled, and mixed with equal volumes of a stabilizing medium before storage at −70 C. This freeze diluent consisted of 0.75% BA in 0.12 M NaCl-0.05 M H_3BO_3 at pH 9.0. Then 2.5 ml of freeze diluent was added to each of the same three petri plates. The cells were lysed by freeze-thawing and the fluids were pooled for assay of total virus. Both free and total virus production were calculated on the basis of PFU per cell.

Viral neutralization. The rate of neutralization of Modoc virus by hyperimmune hamster sera and the influence of complement on the neutralization were studied. Two suspensions of virus, each containing approximately 10^6 PFU/ml, were prepared in PBS. One viral suspension contained 20 U of guinea pig complement per ml. Equal volumes of a 1:10 dilution of heat-inactivated normal or hyperimmune hamster serum were mixed with each of the above viral suspensions. The viral suspensions and sera were incubated in a 37 C water bath before and after mixing. At selected intervals during a 1-h incubation period, 0.1-ml volumes were collected from each of the four serum-virus mixtures and immediately diluted 1:100 in PBS that contained 0.75% BA and placed in an ice bath. Residual infectious virus was titered by plaquing on Vero cells.

Thermal inactivation. The thermal inactivation rates of Modoc virus were determined at 7, 22, and 37 C. For this experiment a suspension of virus that contained an estimated 10^6 PFU/ml was prepared in PBS. An estimated 0.5% FCaS was present in the suspension as a carry-over from the stock virus that contained 50% heat-inactivated FCaS and which was diluted 100-fold. Portions of each viral suspension were incubated at each of the three temperatures. Samples (0.3 ml) were removed at various intervals from each viral suspension and added to 2.7 ml of freeze diluent for subsequent assay of infective virus.

RESULTS

Plaque formation in Vero cells. Distinct plaques were apparent in cultures of Vero cells at 6 to 7 days after inoculation with Modoc virus. The diameters of individual plaques ranged from 0.5 to 2 mm. Groups of viable cells often were observed in the center of individual plaques, which gave the plaques a characteristic

donut-like appearance. Plaque formation was prevented by specific immune sera.

The quantitative relationship between adsorption time and the number of plaques that developed is shown in Fig. 1. Maximal adsorption occurred within 90 min of incubation at 37 C.

The dose-response curve of Modoc virus in Vero cells revealed a linear relationship between the dilution of virus inoculated and the number of plaques that developed (Fig. 2). This indicated that a single infectious Modoc viral particle might initiate a plaque.

Simultaneous titrations of the stock brain suspension of Modoc virus in Vero cells and in SM demonstrated that the plaque assay system was as sensitive as ic inoculation of SM for assay of virus. The titers in Vero cells and in SM were 7.8×10^8 PFU and 10^9 SM intracerebral lethal $dose_{50}$ per g of brain, respectively.

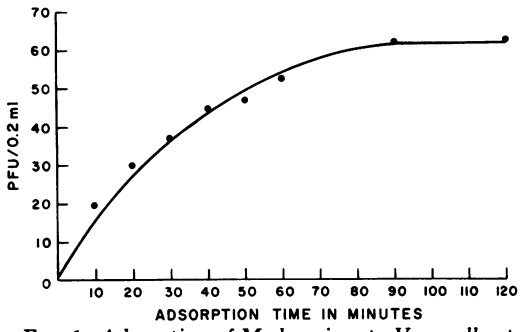

FIG. 1. *Adsorption of Modoc virus to Vero cells at 37 C.*

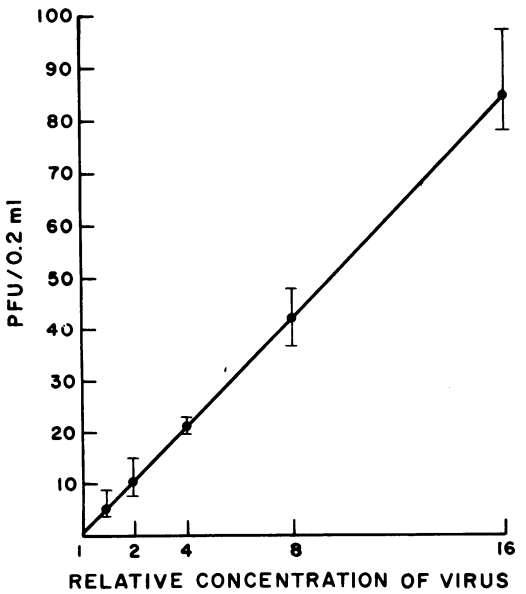

FIG. 2. *Linear dose response of Modoc virus in Vero cells. Symbols: ●, mean of titers; I, range of titers.*

Virus growth. The growth curve of Modoc virus in Vero cells is shown in Fig. 3. There was an initial latent period of approximately 20 h, after which there was an exponential increase in viral titer for the next 20 h. During the remainder of the 83-h period of observation, there was a gradual continuous increase in viral titer. The titer of total virus detectable in the cells exceeded free or released virus throughout the experiment. By 83 h after infection, approximately 8,000 PFU of virus were detectable for each cell in a culture.

Viral neutralization. The $\log_{10}$ neutralization indexes of undiluted and a 1:40 dilution of hyperimmune hamster serum was 7.9 and 2.2, respectively, when the virus-serum mixture was not diluted before assay for unneutralized virus. However, when the virus-serum mixture was diluted 1:100 as required for the kinetic neutralization test, the $\log_{10}$ neutralization index obtained with the 1:40 dilution of hyperimmune hamster serum was only 1.0. Thus, a 1:10 dilution of hyperimmune hamster sera had to be used in the kinetic neutralization test to obtain a $\log_{10}$ neutralization index of 1.6 to 1.8.

The neutralization kinetics of Modoc virus in a 1:10 dilution of hyperimmune hamster serum with and without 20 U of complement are presented in Fig. 4. Maximal neutralization of virus occurred within 45 min of incubation at 37 C. The addition of complement had little effect on the rate of neutralization.

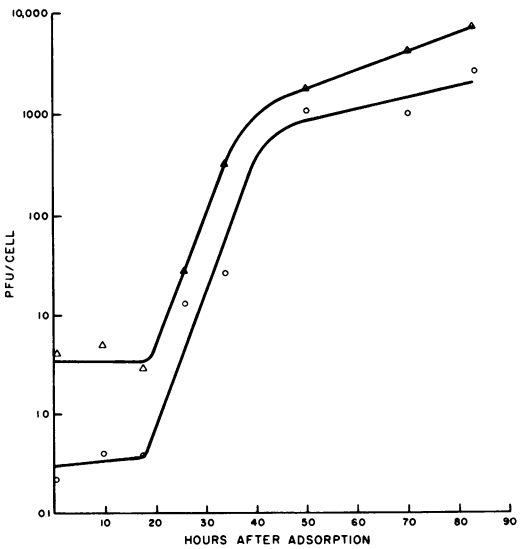

FIG. 3. *Growth curve of Modoc virus in Vero cells infected at a multiplicity of one to two. Symbols: Δ, total virus; O, free virus.*

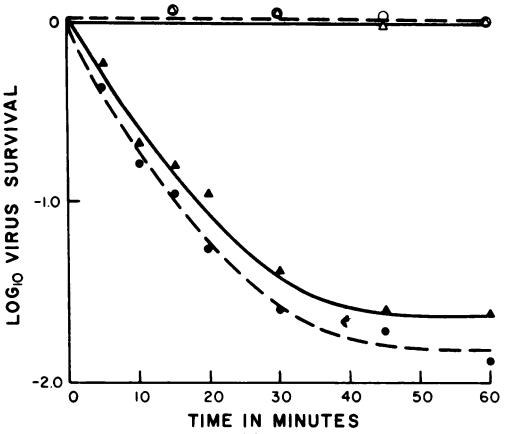

FIG. 4. *Neutralization kinetics of Modoc virus in an hyperimmune hamster sera with and without the addition of 20 U of guinea pig complement. Symbols: Δ, normal hamster sera; O, normal hamster sera supplemented with 20 U of complement; ▲, 1:10 dilution of hyperimmune hamster sera; ●, 1:10 dilution of hyperimmune hamster sera supplemented with 20 U of complement.*

Thermal inactivation. The stability of Modoc virus at 7, 22, and 37 C was determined as a prelude to studies of horizontal transmission of the virus in experimental animals. Virus was not inactivated measurably after incubation for 72 h in PBS at 7 or 22 C (Fig. 5). Incubation for the same time at 37 C resulted in a $10^{2.0}$ to $10^{3.0}$ reduction in titer.

DISCUSSION

Although plaquing of Modoc virus in Vero cells has been reported (9), no attempt was made to determine whether this technique was a sensitive and quantitative system for assay of Modoc virus. The present study demonstrated that plaquing in Vero cells maintained under nutrient methyl cellulose medium was as sensitive as ic inoculation of suckling mice for the assay of a low laboratory-passaged strain of Modoc virus. The linear relationship between the dilution of virus and the number of plaques further indicated that the plaque assay system could be used for quantitative measurements of Modoc viral titer.

Stim (9) found that Modoc virus produced plaques within two days of incubation at 37 C in Vero cells that were maintained under a nutrient medium containing diethylaminoethyl (DEAE)-dextran and Ionagar no. 2. By using a low mouse-passage level of Modoc virus, Hardy (unpublished data) was unable to plaque this virus in Vero cells that were maintained under nutrient medium containing purified agar or agarose with or without DEAE-dextran. When methyl cellulose was used in place of agar, the Modoc virus produced distinct plaques in Vero cells, but these plaques were not apparent until 5 or 6 days after infection and were not countable until 7 days. The exact reasons for these discrepancies in plaquing Modoc virus in Vero cells cannot be determined because the conditions for culturing cells, original source of cells, and passage level of the virus were also different in each laboratory.

The rate of multiplication of Modoc virus in Vero cells was relatively slow. After an initial latent period of about 20 h, infectious virus was continually released from cells for over 60 h. Total virus in the cell culture exceeded free virus throughout the growth period, which indicated that some virus remained cell-associated after maturation. This interpretation is compatible with electron microscope observations made on cells infected with other group B arboviruses (6, 7). In these studies it has been shown that group B arboviruses mature by budding through the intracytoplasmic membranes into intracellular vesicles. Thus, intracellular maturation of Modoc virus in Vero cells could explain the detection of higher titers of total than of free virus. However, the titer of free virus might have been decreased by adsorption of virus onto Vero cells after being released.

Modoc virus was readily neutralized with hyperimmune hamster serum. The addition of guinea pig complement did not enhance the neutralization. Other investigators (2, 12) reported similar findings with herpesvirus where viral neutralization by late (hyperimmune) rabbit serum was not increased by complement. They found, however, that complement did potentiate the neutralization of herpesvirus by early immune rabbit serum.

An interesting finding was that the infectivity of most of the virus rendered noninfectious by incubation with specific antisera was recovera-

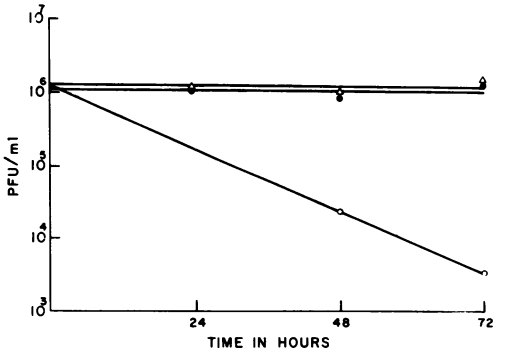

FIG. 5. *Thermal stability of Modoc virus in PBS at 7, 22, and 37 C. Symbols: Δ, 7 C; ●, 22 C; O, 37 C.*

ble when the virus-serum mixture was diluted 100-fold. If a similar situation exists in vivo, then potentially infectious virus might be circulating in complexes with antibody in the blood of hamsters chronically infected with Modoc virus. If this is true, then these complexes could be filtered out by the kidney and account for the viruria in chronically infected hamsters (4).

Modoc virus was quite stable over a 3-day period at 7 and 22 C in PBS at pH 7.4, but was slowly inactivated at 37 C. Provided that Modoc virus is equally stable in urine or other body excretions to thermal inactivation, then horizontal transmission from infected to uninfected animals might occur in nature as suggested by Johnson (4).

ACKNOWLEDGMENTS

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