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A Rapid Plaque Assay for Japanese Encephalitis Virus Infectivity by the Presence of Actinomycin D in Overlay Medium

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Abstract: An improved rapid plaque assay for Japanese encephalitis (JE) virus infectivity was developed. When Actinomycin D was introduced at 0.2 μg/ml concentration into the overlay medium which contained 2% fetal calf serum and 1% methyl cellulose in Eagle’s medium, clear plaques of Nakayama strain JE virus were formed 52 hr postinfection on BHK21 cells. The improved method provided reasonably rapid result of virus infectivity with less labor and cost than conventional plaque or focus assay.

Key words: Japanese encephalitis virus, Plaque formation, BHK21 cells, Actinomycin D

INTRODUCTION

Japanese encephalitis (JE) is characterized by high case fatality and grave sequelae, and is present in several Asian countries. Its causative agent, JE virus is a member of genus flavivirus family Flaviviridae and was formerly classified as group B arbovirus, because of its transmission cycle and antigenic cross-reactivity. Infectivity assay of the virus is prerequisite in the basic as well as applied virology, and most routinely and accurately be performed by plaque formation on virus-sensitive cell culture. In the case of JE virus, Inoue et al. (1961) adopted the plaque assay on primary chick embryo cell culture which was developed by Dulbecco and Vogt (1954) by using agar overlay. DeMadrid and Porterfield (1969) and Hashimoto et al. (1971) used PS cells with carboxymethyl cellulose overlay on microplates for the plaque assay of group B arboviruses including JE virus. In our Department, we have been using Hashimoto’ method modified to semimicroplate, BHK21 cells and methyl cellulose overlay medium (Shameem et al., 1989). These methods are simple and reliable but time-consuming, usually 4-5 days. Many efforts have been made to cut-off the time in order to get rapid results, for example, focus formation by staining virus-infected cells using immunoperoxidase method (Okuno et al., 1985). This method, although providing rapid results, required several steps and reagents of immunostaining. Actinomycin D has long been know.
as a potent inhibitor of DNA-dependent RNA polymerase, and does not usually inhibit growth of RNA viruses, including JE virus. During our studies on the growth of JE virus, we found that Actinomycin D at certain concentration enhanced the virus yield in BHK21 cells and reduced the time of plaque formation when introduced into the overlay medium.

MATERIALS AND METHODS

Cells: *Aedes albopictus*, clone C6/36 cells (Igarashi, 1978) were used to prepare the seed of JE virus. BHK21 cells were used for growth and plaque assay of JE virus and grown with 9% fetal calf serum (FCS) in Eagle’s medium. The cell suspension at a concentration of $2.5 \times 10^5$ cells/ml was seeded onto 12 well plates (2 ml/well) or 24 well plates (1 ml/well), and incubated at 37°C in 5% CO₂-atmosphere to form monolayers.

Virus: Nakayama strain of JE virus was used throughout this study. Seed virus was harvested from infected C6/36 cells, divided into aliquots and kept at −70°C for later use.

Actinomycin D: The product from Sigma Chemical Company No. A-4262 was used. The stock solution of 500 μg/ml was diluted to a working solution of 50 μg/ml, and introduced into the overlay medium or maintenance medium at required final concentrations.

Virus growth experiment: Growth medium was removed by aspiration from BHK21 cells in 24 well plates and 0.1 ml of seed virus was inoculated to each well at appropriate dilution to contain $10^6$ PFU/ml. Virus adsorption was carried out for 2 hr with spreading the inoculum every 30 min at 37°C in 5% CO₂-atmosphere. Residual virus was removed and cells were washed twice with PBS. The cells were covered by 1 ml/well of the maintenance medium with different concentrations of Actinomycin D, and were incubated at 37°C in 5% CO₂-atmosphere. The infected fluid was harvested at different intervals from duplicate wells and kept at −70°C. Virus infectivity of each specimen was titrated by focus counting according to Okuno’s method (Okuno et al., 1985).

Plaque assay: The seed virus was serially diluted in 10-fold steps with 0.2% gelatin in PBS. The growth medium was removed from monolayer culture of BHK21 cells by aspiration, and diluted virus was inoculated using 0.2 ml/well for 12 well plate or 0.1 ml/well for 24 well plate. After 2 hr adsorption at 37°C in 5% CO₂-atmosphere, spreading the inoculum every 30 min, the cells were covered with overlay medium containing 1% methyl cellulose, 2% fetal calf serum in Eagle’s medium with varying concentration of Actinomycin D. The cells were incubated at 37°C in 5% CO₂-atmosphere for appropriate period. The overlay medium was removed and cells were rinsed with PBS, fixed with cold methanol at −20°C for 30 min, rinsed with tap water and stained with 1% crystal violet solution in ethanol for several min. Excess dye was removed by rinsing with tap water and plates were air-dried to count the number of the plaques.
RESULTS

The effects of Actinomycin D at different concentrations on the growth of Nakayama strain of JE virus in BHK21 cell

Fig. 1 shows yield of JE virus infectivity from infected BHK21 cells in the presence of varying concentration of Actinomycin D. The virus yield 1 day after infection was higher with 0.2 or 1.0 µg/ml Actinomycin D than the control without Actinomycin D or with 5.0 µg/ml Actinomycin D. While, the presence of Actinomycin D in the maintenance medium appeared to reduce the virus yield 2 days after the infection.

In order to see the effect of Actinomycin D on the growth of JE virus more in detail, the infected BHK21 cells were incubated with the maintenance medium with or without 0.2 µg/ml Actinomycin D and virus infectivity in the infected culture fluid was assayed every 6 hr after the infection. The result in Fig. 2 showed that virus infectivity obtained in the presence of Actinomycin D was higher than the control without Actinomycin D from 24 to 42 hr after infection. While, the virus infectivity obtained with Actinomycin D was less than that of the control 60-72 hr after the infection.

These data suggested that Actinomycin D at certain concentration could somehow enhance the virus yield in BHK21 cell.

Determination of the optimal concentration of Actinomycin D for PFU assay

Fig. 3 shows plaques of JE virus formed on BHK21 cells in the presence of varying

Fig. 1. Effects of Actinomycin D at different concentrations on the growth of Nakayama strain JE virus in BHK21 cell. The infected BHK21 cells were incubated with maintenance medium containing Actinomycin D at final concentration of 0, 0.2, 1.0, and 5.0 µg/ml, and infected culture fluid was harvested 1 day (■—■) or 2 days (△—△) after the infection to assay virus infectivity as described in the Materials and Methods.
concentration of Actinomycin D in the overlay medium. Actinomycin D at a concentration of 0.5 \( \mu g/ml \) in the overlay medium appeared to be too toxic to BHK21 cells resulting in the cell destruction (line C). While, in the presence of less concentration of Actinomycin D in overlay medium (0.25 \( \mu g/ml \) in line B or 0.125 \( \mu g/ml \) in line A), clear plaques were formed 2 days after the virus inoculation. Since the plaques in line B were larger and clearer than in line A, 0.2 \( \mu g/ml \) Actinomycin D at final concentration was chosen to perform plaque assay of JE virus.

![Growth curves of Nakayama strain JE virus in BHK21 cell with or without Actinomycin D in the maintenance medium. Infected BHK21 cells were incubated in the maintenance medium with (△ − △) or without (■ − ■) 0.2 \( \mu g/ml \) Actinomycin D and virus infectivity in the infected culture fluid was assayed at 6 hr interval.](image)

![Effect of Actinomycin D in the overlay medium on the plaque formation of JE virus on BHK21 cells. Seed virus was diluted in 2-fold steps starting from 10\(^{-3}\) and inoculated into BHK21 cells on 24 well plates to form plaques under methyl cellulose overlay medium containing Actinomycin D at final concentration of 0.125 \( \mu g/ml \) in line A, 0.25 \( \mu g/ml \) in line B, and 0.5 \( \mu g/ml \) in line C, respectively.](image)
Determining the optimal time for PFU assay in the presence of 0.2 μg/ml concentration of Actinomycin D

Fig. 4 shows the infectivity of Nakayama strain JE virus determined by plaque assay at different incubation time in the presence of 0.2 μg/ml Actinomycin D in the overlay medium. The plaques were too small at 48 hr after infection, large and clear enough at 52 and 56 hr at appropriate virus dilution, and became larger but confluent at 60 hr of incubation. Because the virus infectivity at 52-60 hr after infection was almost the same, the incubation time between 52 to 56 hr after infection was suitable for plaque assay for Nakayama strain JE virus on BHK21 cell.

Fig. 5 shows JE virus plaques on BHK21 cells in 12 well plate 52 hr post inoculation. The line A and C with 0.2 μg/ml Actinomycin D showed clear plaques, while plaques on line B without Actinomycin D were too small to count.

Fig. 6 is the relationship between virus dilution and number of plaques per well obtained 52 hr after infection in the presence of 0.2 μg/ml Actinomycin D in the overlay medium. The linear relationship indicates the validity of this plaque assay method for the infectivity titration of JE virus. The least squares regression line was calculated as \( Y = -5.69X + 53.24 \), where \( X \) is logarithm of dilution, and \( Y \) is the number of plaques.

Fig. 4. Infectivity titer of JE virus determined at different incubation period for plaque formation in the presence of 0.2 μg/ml Actinomycin D in the overlay medium. The infectivity of the seed virus was assayed by plaque formation in the presence of 0.2 μg/ml Actinomycin D in the overlay medium and plaques were stained at 48, 52, 56 and 60 hr after infection.
Fig. 5. Plaques of Nakayama strain JE virus formed on BHK21 cell with or without 0.2 \( \mu \)g/ml Actinomycin D in the overlay medium. BHK21 cells on 12 well plate were inoculated with seed virus diluted to \( 10^{-3} \) (column 1), \( 10^{-4} \) (column 2), and \( 10^{-5} \) (column 3 and 4). After the adsorption period, the cells were covered with overlay medium containing 0.2 \( \mu \)g/ml Actinomycin D (line A and C) or without Actinomycin D (line B).

Fig. 6. Relationship between the virus concentration (X-axis) and the number of plaques formed under the overlay medium containing 0.2 \( \mu \)g/ml Actinomycin D (Y-axis in log scale). The least squares regression line (\( Y = -5.96X + 53.24 \)) is shown.
DISCUSSIONS

As shown in this paper, plaque formation of the Nakayama strain JE virus was accelerated by the presence of 0.2 μg/ml Actinomycin D in the overlay medium. The principle of plaque formation is to differentiate accumulated dead cells by virus infection from surrounding surviving cells. If the virus growth is accelerated by Actinomycin D, the plaque formation will be enhanced. Mallo et al. (1991) reported that 0.5 μg/ml of Actinomycin D stimulated avian reovirus growth in L cells suggesting that translational competition dictates the fate of the infection because Actinomycin D inhibits cellular RNA polymerases I and II at concentrations which do not affect the activity of virus-coded RNA-directed RNA polymerases. In the case of JE virus, the virus yield in the infected culture fluid was increased by Actinomycin D. The result, however, cannot simply be explained as the result of enhanced virus multiplication by Actinomycin D, and may be the result of increased release of the infective virus into the medium due to cellular damage by the drug.

Compared with other plaque assays for JE virus, our method has 2 advantages. One is the reduced incubation time of 2 days to get the results compared with 4-5 days by previous methods, reducing the chance of bacterial contamination. Secondly, our method does not require several steps and reagents which were used in the focus formation and immunostaining (Okuno et al., 1985), therefore less expensive. When the same seed virus was assayed by Okuno's method and our improved plaque method, almost similar titer was obtained by both methods. Therefore, this improved plaque assay method will be utilized for future studies on JE virus which require infectivity assay of the virus.

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REFERENCES


