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Effects of Cell Culture and Laboratory Conditions on Type 2 Dengue Virus Infectivity

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The stability of type 2 dengue virus to exposure to a variety of laboratory conditions was determined. Suckling mouse brain passage virus was adapted for growth in BHK-21 cells, and plaque assays were performed using a tragacanth gum overlay. A three- to fourfold increase in plaque size could be obtained if monolayers were subconfluent at time of inoculation. Incubation of virus for 24 h at 37°C, pH 6.5, or in buffer containing 1 mM ethylenediaminetetraacetate considerably reduced virus infectivity as compared with virus incubated for the same period at 4°C, pH 8.0, or in buffer with or without 1 mM $CaCl_2$ and 1 mM $MgCl_2$. Multiple freezing and thawing of virus in tissue culture medium containing 10% fetal calf serum did not reduce virus infectivity.

Dengue virus (DEN), an arthropod-borne flavivirus, is responsible for benign dengue fever as well as life-threatening dengue hemorrhagic fever and dengue shock syndrome. The virus has been reported to replicate in cell cultures derived from a number of different species (3–5, 18–22), and several techniques for the isolation and serological identification of the virus, as well as laboratory techniques for biochemical studies of the agent, are based upon cell culture methods (1, 2, 4, 6, 10, 16, 22).

Although conditions for the plaque assay of DEN under agar and starch have been reported (11, 13–15, 17), comprehensive and detailed information on the stability of the virus to common cell culture and laboratory environments is limited. The present paper describes the properties of the virus grown in BHK cells and the conditions for use of a tragacanth gum plaque assay which allows for readily discernible plaques. Studies on DEN type 2 (DEN-2) held at temperatures of 4 and 37°C (pH 6.5 through 8.0), diluted in buffers varying in divalent cation concentration, or subjected to multiple freeze-thaw cycles, as presented here, are intended to characterize the biostability of the virion when subjected to conditions encountered in the laboratory.

MATERIALS AND METHODS

Cells. BHK-21 clone 15 (BHK-21/15) cells were kindly provided by Joel Dalrymple (Walter Reed Army Institute of Research). The cells were grown as monolayers in 75- or 25-cm^2 plastic flasks. The growth medium consisted of Eagle minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS), 100 U of penicillin per ml, and 100 μg of streptomycin per ml.

Virus. DEN-2, New Guinea C strain, suckling mouse brain passage 35, was kindly provided by Walter Brandt (Walter Reed Army Institute of Research) as a 20% suckling mouse brain suspension. The suckling mouse brain suspension was inoculated onto BHK-21/15 cells, and the virus was propagated in monolayer cell cultures as described in the text.

Virus infectivity. DEN-2 infectivity was determined by plaque assay in BHK-21/15 cells by using a tragacanth gum overlay method similar to that described previously for several other animal viruses (9). Virus samples were diluted with serum-free MEM and inoculated onto BHK-21/15 cell monolayers in 25-cm^2 plastic flasks. The virus was allowed to adsorb for 90 min at 37°C. Flasks were rocked at 10-min intervals to redistribute the inoculum. Monolayers were then washed three times with serum-free MEM. Six milliliters of 0.75% tragacanth gum, in MEM containing 10% FCS and antibiotics as noted above, was added. The cultures were incubated at 37°C for 5 days, after which the gum was removed and the monolayers were stained with crystal violet.

RESULTS

Plaque assay. DEN-2 was titrated by plaque assay on BHK-21/15 cells using a semisolid tragacanth gum overlay. A 5-day incubation period was found to be optimal for obtaining readily detectable plaques. Nonspecific degeneration of the monolayer was not encountered under the assay conditions used. We observed that cell cultures which were 80% confluent, rather than confluent, when inoculated with virus yielded substantially larger plaques. Cultures that were approximately 80% confluent at inoculation yielded plaques ranging in size from 0.5 to 3.5

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mm (Fig. 1A), whereas cultures confluent at inoculation produced plaques ranging from 0.5 to 1.5 mm in diameter (Fig. 1B). A heterogeneity in DEN-2 plaque size as noted in both Fig. 1A and B was a consistent observation. Plaque titer was the same when either confluent or subconfluent cultures were used.

Growth of virus in BHK-21/15 cells. Virus obtained as seed stock (suckling mouse brain suspension) was adapted to BHK-21/15 cells by serial passage. DEN-2 seed stock, 0.5 ml, was inoculated onto cell monolayers in 25-cm^2 flasks. After a 90-min adsorption period the monolayers were washed with FCS-free MEM, and 6 ml of MEM containing 10% FCS was added. Tissue culture fluid (TCF) was removed 72 h postinfection or when cell degeneration was apparent. The TCF was cleared of cells by centrifugation at 2,000 × *g* for 10 min, and a sample of the cleared TCF was inoculated onto a nearly confluent monolayer. After six serial passages of DEN-2 in BHK-21/15 cells, the titer increased from 6×10^3 to 3×10^7 plaque-forming units per ml.

Multiplication cycle of BHK cell-adapted virus. The growth curve of DEN-2 propagated in BHK-21/15 cells was determined. BHK cells were inoculated with virus (passage 6) at a multiplicity of infection of 10, and 0.1-ml volumes of TCF (20-ml initial volume) were harvested at 12-h intervals. The TCF was prepared and assayed for infectious virus as described above. An increase in extracellular DEN-2 was detected in the medium at 24 h postinfection (Fig. 2). Maximum titers were found between 36 and 60 h. After 60 h the titer of infectious extracellular virus rapidly decreased.

Stability of infectivity at 4 and 37°C. Since DEN is grown in vertebrate cells in cell culture at or near 37°C and the virus is often stored at 4°C, we examined the effect of these temperatures on survival of infectivity (Fig. 3). Samples of TCF from virus-infected cells which had been clarified by low-speed centrifugation were placed into small vials and sealed. The vials were held at either 4 or 37°C for different intervals. Vials were stored at −70°C for plaque assay.

Virus maintained in TCF at 4°C for 24 h showed no reduction in titer. A reduction of about 40% was detected for virus held at 4°C for 48 h. In contrast, DEN-2 held at 37°C was rapidly inactivated. After incubation at 37°C for 12 h, only 30% of the initial infectivity remained. Less than 0.01% of the infectivity survived for

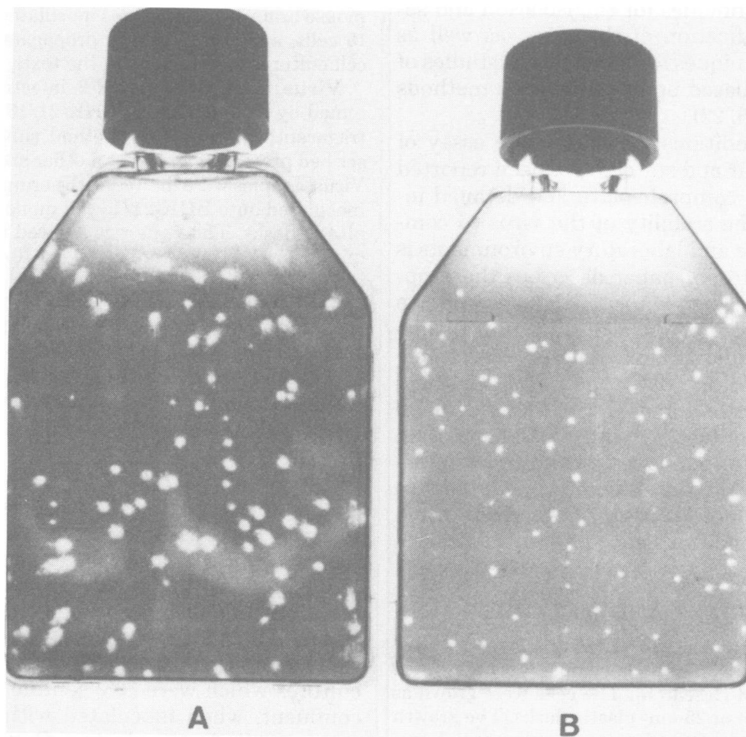

FIG. 1. *Plaques formed by DEN-2 in BHK-21/15 cells on day 5 postinfection. Plaques developed under tragacanth gum. Cultures were inoculated when cells were 80% confluent (A) and totally confluent (B).*

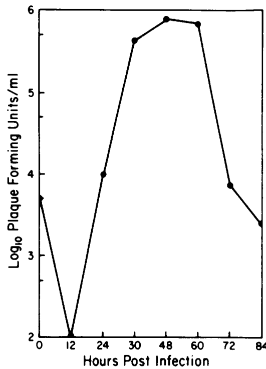

FIG. 2. *Growth curve of DEN-2 in BHK-21/15 cells. Cells at confluency were inoculated with DEN-2 at an MOI of 10.*

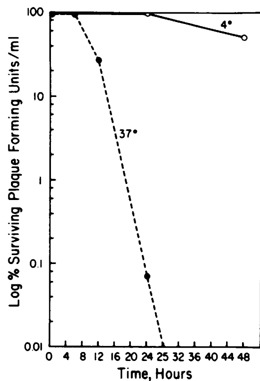

FIG. 3. *Survival of DEN-2 infectivity at 37 and 4°C. Samples of DEN-2 maintained at the given temperatures were assayed for infectivity.*

24 h at 37°C.

Influence of pH. Metabolism of cells in culture may lead to an appreciable shift of the growth medium to acid pH. In serial passage studies we noted that DEN-2 infection of BHK-21/15 cells increased the rate of the shift to acid pH. To determine the effect of pH on DEN-2 infectivity, virus-infected TCF cleared of cells was diluted 100-fold with MEM containing 10% FCS buffered to pH 6.5, 7.0, 7.5, or 8.0. Vials containing the diluted virus were sealed and incubated at 4°C for 24 h. Before plaque assay, all samples were adjusted to pH 8.0. A small but progressive decline in infectivity followed reduction in pH. A 70% loss of infectivity resulted from incubation at 4°C for 24 h at pH 6.5 as compared with virus maintained at pH 8.0.

Influence of divalent cations. Since chelating agents which reduce divalent cation concentration have been used during DEN isolation and characterization, experiments were performed to determine the effect of divalent cations on DEN infectivity. TCF containing DEN-2 was cleared of cells and diluted 1,000-fold in the following solutions: tris(hydroxymethyl)-aminomethane-buffered saline (TBS), pH 8.0; TBS containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$; TBS containing 1 mM ethylenediaminetetraacetate; MEM containing 10% FCS. The diluted samples were placed in vials, sealed, and then held at 4°C for 24 h. All samples were diluted in serum-free MEM (pH 8.0) before plaque assay. DEN-2 infectivity was not substantially changed when diluted in TBS or TBS containing $MgCl_2$ and $CaCl_2$ (Table 1). However, infectivity was reduced by nearly 3 $\log_{10}$ plaque-forming units per ml when the TBS diluent was supplemented with 1 mM ethylenediaminetetraacetate.

Effect of multiple freeze-thaw cycles. DEN isolates are routinely transported and stored at temperatures of −70 to −80°C and are occasionally subjected to several cycles of freezing and thawing. The effect of freezing and thawing cycles on DEN-2 infectivity was examined by assaying virus for infectivity in TCF after several cycles of freezing at −75°C and thawing to 25°C. An ampoule containing a thermocouple immersed in TCF was used to monitor temperatures. DEN-2 infectivity was stable under the test conditions. No reduction in titer was noted after one, two, or three cycles of freezing and thawing.

DISCUSSION

The data presented in this paper describe the stability of DEN-2 grown in BHK-21/15 cells under controlled cell culture and laboratory conditions. In our studies, viral infectivity was monitored by plaque assay under a semisolid tragacanth gum overlay not previously described for

TABLE 1. *Effect of divalent cation removal on DEN infectivity*

Treatment[a]	Plaque-forming units per ml
Untreated[b]	3×10^4
MEM + 10% FCS	2×10^4
TBS[c]	1×10^4
TBS + 1 mM $CaCl_2$ + 1 mM $MgCl_2$	2×10^4
TBS + 1 mM EDTA[d]	30

[a] Virus in MEM was diluted 1,000-fold in the indicated solutions and stored at 4°C for 24 h. For titration, all virus samples were diluted into complete MEM + 10% FCS before infection.

[b] Untreated virus in TCF was diluted 1,000-fold in MEM with 10% FCS and assayed in the same way as test samples.

[c] pH 8.0.

[d] EDTA, Ethylenediaminetetraacetate.

DEN. In developing the plaque assay it was noted that plaque size at the end of the 5-day incubation period was related to the degree of monolayer confluency at time of virus inoculation.

Although DEN plaque assays often yield a range of plaque sizes (13), we noted an appreciably greater range of plaque sizes in cultures which were subconfluent at inoculation when compared with those which were confluent. In a previous study, monkey kidney cell cultures inoculated on day 3 or 24 after transfer showed little difference in plaque titer (14); variation in plaque size was not reported. However, given the cell transfer conditions used, it is likely that cultures were confluent on both days 3 and 24. Increased plaque size in the subconfluent cultures may simply reflect the larger size of cells which upon death give rise to larger plaques. Alternatively, the larger plaque size could be the result of a more efficient replication in subconfluent monolayer cells, thereby increasing virus yield per cell, reducing the latent period, or both; however, virus yield did not increase when subconfluent monolayers were used for virus production.

In an attempt to increase virus yield a polycation, polybrene, was added to the virus inoculum at final concentrations of 1, 2, 4, and 6 μg/ml. Although polybrene has been shown to enhance yields of lipoprotein-enveloped viruses (7, 21), no increase in virus titer was noted when the polycation was added to DEN-2 inocula (data not shown).

The growth curve of DEN-2 in BHK-21/15 cells showed release of the virus by 24 h with peak titers between 36 and 60 h postinfection. The pronounced decrease in titer found in the present study between 60 and 84 h postinfection is most likely due to the combined thermal inactivation of virus released into the medium and to the degeneration of the cells. The temperature sensitivity study demonstrated a reduction of greater than 3 $\log_{10}$ plaque-forming units per ml in 24 h at 37°C (Fig. 2), so thermal inactivation would account for the decline, assuming that virus replication and release was essentially completed by 60 h. In a previous study DEN-2 titer was reported to decline after 1 h at 37°C, and infectious virus was not detected after 12 h (19). This rate of inactivation is somewhat greater than that which we observed; however, the previous report did not give experimental conditions, and the more rapid inactivation may have been due to a combination of factors. Nevertheless, the results of both studies suggest that if maximum virus recovery is to be obtained, virus should be harvested at several times after infection prior to the appearance of maximum cytopathic effect and stored at 4 or −70°C.

The infectivity of DEN-2 was found to be dependent upon the pH of the suspension medium when maintained at 4°C for 24 h. The titer decreased as the pH was reduced (Table 1). This finding points up the importance of maintaining a basic pH when growing and storing the virus. These studies were conducted at 4°C to allow distinction between pH and temperature effects, but the results are undoubtedly applicable to virus released from infected cells at 37°C. It has been previously reported that a variation in pH between 6.6 and 8.6 of the agar overlay in DEN plaque assay had little effect on plaque titer (14) in apparent discrepancy with our findings. However, during plaque formation it is unlikely that virus released from infected cells resides in the matrix medium for periods sufficient to show the pH effects as reported here.

The maintenance of DEN-2 infectivity in solution requires divalent cations, as shown by the dramatic drop in virus titer after incubation in a TBS containing 1 mM ethylenediaminetetraacetate (Table 2). Since there was little change in infectivity upon suspension in TBS, the divalent cations are probably bound to the virion in a relatively stable manner. The ability of ethylenediaminetetraacetate to reduce infectivity may indicate that those essential divalent cations are probably located in the viral envelope near the virion surface and may function in the stabilization of viral envelope proteins, as recently found for feline leukemia virus (R. K. Durbin and J. S. Manning, submitted for publication). Our data suggest that chelating agents should not be used during isolation or characterization of DEN.

The addition of Mg^{2+} and Ca^{2+} to TBS in which DEN-2 was suspended neither enhanced nor reduced virus infectivity. In two previous studies on DEN plaque formation it was reported that variation in Mg^{2+} and Ca^{2+} concentrations was without effect (17) or with slight enhancing effect (14), although the latter may have been due to the inactivating effects of the divalent cations on interfering components in the assay rather than on the virus directly. More recently, the addition of divalent cation to DEN-2-infected Vero cell cultures was reported to result in increased cell-free titers (8; T. Matsumura, V. Stollar, and R. W. Schlesinger, Bacteriol. Proc., p. 190, 1970). The authors suggest that the higher titers recovered from these cultures were due to an enhancement of virus maturation and release. In addition, the enhancing effect was restricted to virus-infected Vero cells and was not observed when infected KB or BHK cells were tested.

It is known that DEN can survive for years

when maintained at −70 to −80°C temperatures (3). When DEN-2 in TCF containing 10% FCS was subjected to three cycles of freezing and thawing there was no detectable change in titer. Our results indicate that field and laboratory samples of DEN routinely stored in serum-containing fluids at −70°C or lower can be subjected to several cycles of freezing and thawing without deleterious effect on infectivity.

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