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Development of Dengue Virus Plaques Under Serum-Free Overlay Medium

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An improved plaque assay for dengue virus was developed utilizing baby hamster kidney (BHK-21) cells initially grown in shaker culture. Different media preparations were tested for uniform and fast formation of BHK-21 cell sheets. Several overlay formulas were tested to develop a rapid plaque assay in 6- and 24-well plastic plates. The best results were obtained utilizing Eagle minimal essential medium (pH 7.2 to 7.4) supplemented with 1 mg of $NaHCO_3$ per ml and 5% newborn calf serum for the formation of cell monolayers after 8 to 24 h of incubation at 37°C. Serum-free Eagle minimal essential medium supplemented with 1% methylcellulose and buffered with 10 mM *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid (pH 7.4 to 7.6) was used as an overlay medium. This system allowed for plaque formation after 3 days of incubation of dengue type 2 virus and after 4 days for dengue type 1 and 4 viruses.

Quantitative assays in cell culture to titrate dengue virus infectivity have been reported by many investigators (for reviews, see 9, 19). A disadvantage of these methods is the long incubation time (6 to 14 days) required for optimum plaque development (4–6, 10, 15–26). Also, large numbers of cells needed for the assay must be grown in monolayer cultures, requiring the use of large amounts of media and sera (4–6, 10, 15–26). Moreover, preparation of cells for the assay from cell monolayers requires the use of trypsin or Pronase, which can have some deleterious effects on cells (14, 27). Recently, the immunoperoxidase (13) and fluorescent-antibody (11) techniques have been introduced to improve dengue virus titration. These methods decrease the time required for dengue virus titration to 3 days, but they require specific antisera and special equipment.

This report presents a simple plaque assay for dengue virus titration for which results are obtained within 3 or 4 days. Baby hamster kidney (BHK-21) cells grown in shaker culture were used as a source of cells, and a single serum-free overlay medium was used for plaque development.

MATERIALS AND METHODS

Virus. Dengue virus type 1 (Hawaii strain), dengue virus type 2 (D2) (New Guinea C strain) and dengue virus type 4 (H-241 strain) were obtained from P. K. Russell and W. Brandt (Walter Reed Army Institute of Research, Washington, D.C.). Dengue virus type 1 (Mochizuki strain) was obtained from S. Makino and S. Hotta (Kobe University Medical School, Kobe, Japan). All of these strains were received as 10% mouse brain homogenates. The viruses were further propagated in mouse brain (9), as well as serially cultivated in BHK-21 cells using a shaker culture system (7).

Cell cultures. BHK-21 cells obtained from T. B. Stim (Yale Arbovirus Research Unit, Yale University, New Haven, Conn.) were cultivated in a shaker culture system and subcultured according to the method of Guskey and Jenkin (7). To prepare cells for the plaque assay, BHK-21 cells were grown in shaker culture for 48 to 72 h at 37°C and then centrifuged for 10 min at 500 × *g* at 22°C. Cells were usually resuspended to obtain 2 × 10^5 cells per ml in a modified Eagle (3) minimum essential medium (MEM) containing Hanks salt solution or in Waymouth medium 752/1 (28) (Grand Island Biological Co., Grand Island, N.Y.). Media were supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml). Serum and other components were added to the cell suspension, depending upon the experimental conditions. Cells were dispensed with a Cornwall syringe, adding 0.5 or 2.5 ml into each of 15- or 35-mm-diameter wells, respectively (Linbro or Costar 24- and 6-well plates). Plates were incubated at 37°C in a CO_2-humidified incubator.

Overlay medium. The overlay medium consisted of MEM supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml). Supplementation of the medium with newborn calf serum (NBCS), methylcellulose (MC)–15 cps (Methocel A-15, Dow Chemical Co., Midland, Mich.), *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid (HEPES), and other components was varied as described below to determine the optimal conditions for plaque development.

Virus plaque assay. BHK-21 cell monolayers (in 15- or 35-mm wells) were washed once with Hanks balanced salt solution just before use, and 0.1 ml of

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virus inoculum was absorbed, generally for 60 min at 37°C. Plates were agitated every 15 min to aid virus-cell interaction and to prevent drying of the cell sheets. Balanced salt solution supplemented with 0.4% bovine serum albumin fraction V (Miles Laboratories, Kankakee, Ill.) at pH 7.8 was used as a virus diluent (21). After infection, 0.5 and 2.5 ml of the overlay medium were added to each of 15- and 35-mm wells, respectively. The plates were incubated for 3 to 8 days as described above. The overlay medium was removed, and cell monolayers were washed once with balanced salt solution and stained with 2% crystal violet according to Holland and McClaren (8).

RESULTS

Cell sheet formation. The optimal amount of NBCS in the medium (MEM or Waymouth) was determined as 5 or 10%. Higher concentrations of serum, i.e., 15 or 20%, caused cell clumping, and uniform monolayers were not obtained. Less than 5% serum was not sufficient for good cell attachment to the plastic surface.

The pH and concentration of sodium bicarbonate in the growth medium influenced the quality and time of formation of BHK-21 cell monolayers (Table 1). The most rapid monolayer development occurred at pH 7.2 in medium containing 1 mg of $NaHCO_3$ per ml. The optimal volumes for cell suspensions were 0.5 and 2.5 ml for 15-mm (1.8-cm^2 surface) and 35-mm (9.6-cm^2 surface) wells, respectively. When smaller volumes were used, the cell monolayer showed a heavier cell sheet on the periphery of the well than in the center. The optimum range of numbers of cells to be added to the wells was 5×10^4 to 7×10^4 per cm^2. The addition of large numbers of cells had the same effect as using high concentrations of serum: the cells aggregated before attaching to the surface. The use of lower cell numbers did not prevent uniform monolayer formation but increased the time required to produce a confluent monolayer.

Cell-virus infection time. The cell monolayers formed at varying times of incubation (Table 1) were tested for their susceptibility to D2 virus infection. One- and 2-day-old BHK-21 monolayers were washed once with balanced salt solution and infected with 0.1 ml of D2 inoculum containing about 10, 50, or 100 plaque-forming units (PFU)/well. After incubation at 37°C for 30, 60, 90, 120, and 150 min, the medium from three plates (18 wells) was aspirated and 2.5 ml of overlay medium was added (see below). After 6 days of incubation at 37°C, the cell sheets were stained and plaques were counted (Fig. 1). Maximum numbers of plaques were obtained after virus absorption for ≥60 min when 50 or 100 PFU were added per 35-mm-diameter well. When 10 PFU/well were introduced, there were no differences in plaque numbers obtained during a virus absorption period of 30 to 150 min. No significant differences of cell susceptibility to D2 virus were observed for 24- or 48-h-old cell monolayers. Based on these data, cell-virus absorption for 60 min was used routinely in all subsequent experiments.

TABLE 1. *Effect of pH and sodium bicarbonate on formation of monolayers of BHK-21 cells*[a]

Sodium bicarbonate concn (mg/ml)	Formation of cell monolayers in medium of different pH levels (h)		
	7.2	7.4	7.6
0.5	12–24	18–36	48–72
1.0	8–12	12–24	24–48
1.5	8–18	12–18	36–48
2.0	12–24	12–24	36–48

[a] BHK-21 cells obtained from shaker culture were resuspended in Eagle MEM containing 5% NBCS. A 2.5-ml volume containing 5.5×10^5 cells was added to 35-mm-diameter wells.

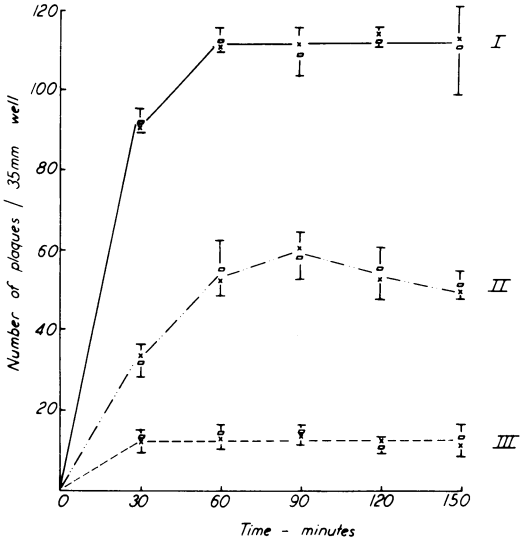

FIG. 1. *Effect of time on virus absorption at 37°C. D2 virus was diluted in balanced salt solution with 0.4% bovine serum albumin, pH 7.8, and 0.1 ml, containing approximately 100 (I), 50 (II), or 10 (III) PFU, was inoculated per well. At the time indicated, the inoculum was removed and overlay medium was added to the wells. Vertical bars indicate maximal deviation from mean counts calculated for 12 wells. Symbols: ×, mean number obtained from six wells with 24-h BHK-21 cell monolayers; □, mean number obtained from six wells with 48-h BHK-21 cell monolayers.*

Optimal conditions for dengue plaque development. The effects of HEPES buffer, MC concentration, and pH of the overlay medium on D2 plaque formation were determined (Table 2). Cell monolayers in 35-mm-diameter wells

TABLE 2. *Effect of pH, HEPES buffer, and MC on time of plaque formation of D2 virus*

Overlay medium			Plaque formation (days)
pH	MC (%)	HEPES (mM)	
7.4	2	0	—[a]
	2	10	—
	1	0	—
	1	10	5
7.6	2	0	8
	2	10	7
	1	0	6
	1	10	5
7.8	2	0	8
	2	10	8
	1	0	6
	1	10	6

[a] No plaques were observed at days 4 and 5; on day 6 the cells were detached from the plates.

TABLE 3. *Effect of concentration of NBCS in the overlay medium on plaque formation of D2 virus*[a]

NBCS (%)	Plaque development		Titer (PFU/ml)[b]
	Time (days)	Size (mm)	
2.0	5	0.5–1	7.6×10^7
1.0	4	0.5–1.5	8.0×10^7
0.5	3–4	0.5–1.5	7.8×10^7
0.2	3	0.5–1	8.1×10^7
0.0	3	0.5–1.0	7.9×10^7

[a] BHK-21 cell monolayers were infected with a 0.1-ml inoculum of D2 virus. After 60 min of absorption at 37°C, the overlay medium was added. After 3 through 6 days of incubation of the plates, the cell monolayers were stained with crystal violet and plaques were counted.

[b] Titer was calculated from the number of plaques obtained in six wells of two different 10-fold dilutions of the virus suspension.

were infected with 200 PFU of D2 virus per well, as described above. The MEM overlay medium supplemented with 2% NBCS and 1 mg of $NaHCO_3$ per ml was added after 1 h. The plates were monitored daily for plaque development. Results showed that 10 mM HEPES buffer prevented a rapid pH change during the incubation period and that the time of the D2 plaque development depended on the pH and the amount of MC in the overlay medium. Optimum results were obtained when 1% MC overlay medium was buffered with 10 mM HEPES at pH 7.4 to 7.6. These conditions were used in subsequent experiments.

The serum concentration in the overlay medium also affected the characteristics of D2 plaque development. The experiments were carried out in the same manner as previously described. Decreasing the serum level from 2 to 0.5% in the overlay medium reduced the time required for D2 plaque development (Table 3). Moreover, D2 plaques were also formed without serum in the medium. The absence of serum did not influence the size, clarity, or number of D2 plaques. Figure 2 illustrates the D2 plaques obtained after 3 days of incubation in BHK-21 cell monolayers with serum-free overlay medium. There were no differences in the condition of monolayers after 3 and 4 days of incubation with or without serum. After 5 days of incubation many cells grown with 2% NBCS were detached from the surface, reducing the confluency of the monolayers, as compared to those grown under serum-free medium.

The magnesium (Mg^{2+}) content of the overlay medium can influence dengue plaque development (23). We studied the effect of Mg^{2+} on D2 plaque formation, using a serum-free overlay

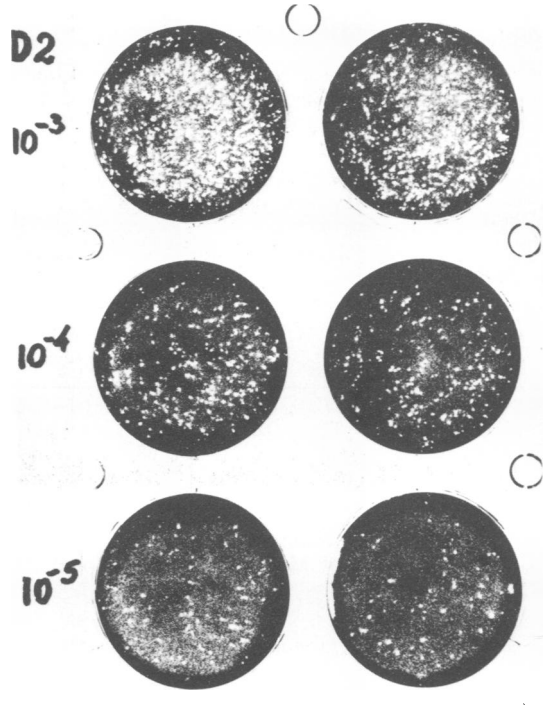

FIG. 2. *D2 plaques of three 10-fold dilutions of virus suspension after 3 days of incubation on BHK-21 cell monolayers with serum-free overlay medium. Crystal violet stain. Size of plate, 82 by 126 mm.*

containing 0.8 to 50 mM Mg^{2+} (Table 4). In the presence of ≥20 mM Mg^{2+} in the overlay medium, the cell monolayers were lighter than those at 0.8 and 10 mM Mg^{2+}. A concentration of 10 or 20 mM Mg^{2+} increased D2 plaque size to some degree. The number of plaques after 3

and 4 days of incubation were about the same using 0.8, 10, or 20 mM Mg^{2+}, but 20 mM Mg^{2+} reduced the clarity of the plaques. The higher Mg^{2+} concentrations (30 and 50 mM) inhibited D2 plaque formation.

Experiments using the optimal conditions found for the rapid D2 plaque assay were repeated with other dengue virus strains: type 1, Hawaii strain; type 1, Mochizuki strain; type 4, strain H-241. These strains were tested for plaque development using an overlay medium with and without serum as described above (Table 5). All dengue virus types formed plaques of similar size and clarity in the presence or absence of serum in the overlay medium. Plaque formation was about 2 to 4 days earlier when the serum-free overlay was used. These data show that dengue virus plaque assay can be expedited by elimination of the serum from the overlay medium.

DISCUSSION

Plaque assays for dengue virus have been performed using cells grown in monolayers (19).

TABLE 4. *Effect of magnesium in the overlay medium on plaque development of D2 virus*

$MgSO_4 \cdot 7H_2O$ in overlay medium (mM)	Plaque development			
	3 days		4 days	
	No. of plaques[a]	Size (mm)	No. of plaques[a]	Size (mm)
0.8[b]	37	0.5–1.0	36	0.5–1.0
10	37	0.5–1.5	39	1.0–2.0
20	31	1.0–1.5	33	1.0–1.5
30	16	0.5–1.5	21	0.5–1.5
50	3	0.5–1.5	7	0.5–1.5

[a] Average plaque count from six wells.
[b] Concentration of $MgSO_4 \cdot 7H_2O$ in Eagle MEM formula (2).

We improved the assay by using BHK-21 cells grown in a shaker culture system (7). The method allows easy production of large numbers of cells which are required for plaque assay. For example, one shaker culture with 50 ml of medium yields 1.4×10^8 cells (7). The same amount of cells can be obtained from 10 monolayer cultures using 350 ml of medium. Cells grown in the shaker culture do not require trypsinization and are easily resuspended without clumping. Another advantage to the use of cells from shaker culture is that monolayers for plaque assay can be obtained in a much shorter time. Cells from shaker cultures required 5% serum (NBCS) for monolayer formation in 8 h (Table 1). Generally twice as much serum was used by other investigators to form cell monolayers for plaque assay within 1 to 4 days (4–6, 20, 22). These differences could be attributed to the detrimental effect of trypsinization of monolayers (27) and to the adaptation of the cells to different serum conditions during cultivation, i.e., 2.5% serum in shaker cultures (7) and ≥10% in monolayers (4–6, 15, 22).

Cell susceptibility to D2 virus was independent of the age of the monolayer (Table 1), which agrees with data obtained by Schulze and Schlesinger for KB cells (20). However, Yuill et al. found some variation in D2 multiplication in monolayers of LLC-MK_2 cells 1 to 4 days old (29). Other authors used cell monolayers grown for different periods of time (1 through 4 days) but did not report the differences in sensitivity of the cells to virus infection (5, 6, 10, 15, 22). BHK-21 cell susceptibility to dengue virus infection under experimental conditions allowed us to obtain quantitative results, using a plaque assay, within 3 days for D2 and within 4 days for dengue virus type 1, Hawaii and Mochizuki strains and dengue virus type 4, strain H-241. This assay permits titration of dengue viruses 2 or more days faster than has been reported by other investigators (2, 6, 10, 17–24, 26).

TABLE 5. *Plaque formation by various dengue virus types in BHK-21 cells using overlay medium with and without NBCS*

Dengue virus strain	Overlay with 2% NBCS			Overlay without serum		
	Titer (PFU/ml)[a]	Plaque		Titer (PFU/ml)	Plaque	
		Size (mm)	Time (days)[b]		Size (mm)	Time (days)
Type 1 (Hawaii)	5.0×10^4	0.5–1	8	5.2×10^4	1.0–1.5	4
Type 1 (Mochizuki)	1.3×10^5	1.0–2	7	1.2×10^5	1.0–2	4
Type 2 (New Guinea C)	1.1×10^8	0.5–1	5	1.3×10^8	0.5–1.0	3
Type 4 (H-241)	2.3×10^6	1.0–2	6	2.0×10^6	1.0–2	4

[a] PFU per milliliter of undiluted virus suspension, calculated from the number of plaques obtained in six wells of two different 10-fold dilutions of the virus suspension.
[b] Times required to obtain the highest number of plaques.

Our results showed that BHK-21 cells can produce dengue virus under a serum-free overlay medium and that the presence of 1 or 2% serum in the overlay medium delayed dengue plaque formation (Tables 3 and 5). These results could have been caused by the inhibitory effect of serum on virus growth. Bentley and Wickham reported a similar effect of serum on influenza A_2 virus (1). Nash and co-workers also reported some inhibitory effect of serum on dengue virus plaque formation, depending on the physicochemical treatment of serum before it was used for plaque assay (16). There could also be an indirect effect of serum on plaque formation due to overgrowth of uninfected cells. In the presence of serum, uninfected cells grew faster than did cells cultivated under a serum-free overlay medium. Such rapid growth could reduce the clarity of plaques formed by the virus.

Decreasing the amount of MC from 2%, commonly used for plaque assay (19, 20), to 1% used for dengue plaque formation on microplate cultures (5) reduced the time required for D2 plaque development. Reduced concentrations of MC to 1% were sufficient to semisolidify the overlay.

The use of 10 mM HEPES buffer was required for stabilizing the pH of the overlay medium during incubation, which aided plaque formation (Table 2). The observed pH dependency for growth of D2 virus, and therefore plaque formation, differed from results obtained by Stim, who did not observe a pH requirement for growth of dengue virus types 1, 2, and 4 in a pH range from 6.6 to 8.6 using LLC-MK_2 cells cultivated in screw-capped bottles (23).

Increasing the Mg^{2+} content up to 20 mM in the overlay medium produced a small increase in D2 plaque size. At $\geq$30 mM Mg^{2+}, an inhibitory effect on plaque numbers was observed (Table 4). These results are different from those obtained by Stim, who found that overlay medium with high concentrations of Mg^{2+} (0.1015 g/ml) and low concentrations of Ca^{2+} (0.0002 g/ml) gave a 0.6-log-higher titer for D2 (New Guinea B strain) virus grown in LLC-MK_2 cells than in an unsupplemented overlay medium (23). The Mg^{2+} concentration used by Stim (0.1015 g/ml = 4.23 M) was more than 100× in excess of the physiological Mg^{2+} concentration for LLC-MK_2 cells (12). Other authors used about 1 mM Mg^{2+} with LLC-MK_2 cells when plaquing dengue viruses (2, 10, 17, 21).

We have described a simple assay procedure for measuring dengue virus infectivity within 3 or 4 days which permits the testing of large numbers of samples in a short period of time. Since a serum-free medium is used, this system can be also employed for studies in which chemically defined conditions are required for dengue virus growth.

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