Dengue Virus Plaque Development in Simian Cell Systems

I. Factors Influencing Virus Adsorption and Variables in the Agar Overlay Medium

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Chemical and physical variables influencing the plaquing of all dengue serotypes in two simian cell systems were studied. Calf serum in the nutrient overlay may be replaced by mouse ascitic fluid or bovine plasma albumin when employing the rhesus monkey kidney LLC-MK2 cell system for plaquing all dengue serotypes. Doubling the serum concentration in the overlay had little effect in modifying dengue types 1, 2, 3, and 4 plaque titers. Newborn agamma, 4-week-old and 8-week-old calf serum gave comparable titers with all dengue virus serotypes. Dengue virus titers, plaque size, and development time were unaffected by sodium bicarbonate concentrations ranging from 1.1 to 4.4 mg/ml of overlay. A twofold increase (0.00332 g%) in the amount of either autoclaved or filtered-sterilized neutral red reduced the dengue 2 virus titer as much as 2.2 logs. An increased Mg++ and decreased Ca++ concentration in the overlay medium increased the efficiency of the plaquing system.

The major problems of in vitro quantitation of all dengue virus serotypes have been surmounted in the past five years (14, 17; T. M. Stevens et al., Bacteriol Proc., p. 132, 1962). Among the many variables influencing dengue virus plaquing efficiency (3, 6, 14, 15, 17), the solidifying agent of the nutrient overlay (agar or methyl cellulose) has been considered the chief factor in the failure or success of this technique with dengue virus.

In this report, factors modifying adsorption of dengue type 2 on primary rhesus monkey kidney cell monolayers have been examined. Nutritional and physiological variables of the nutrient agar overlay affecting plaque development of all dengue virus serotypes in a continuous rhesus monkey kidney cell LLC-MK2 (4) were also studied.

MATERIALS AND METHODS

Viruses. All dengue virus serotypes used in these studies were wet-frozen (−70 C) stocks, prepared as 10% infant mouse brain in a 7.5% solution of bovine plasma albumin in physiological saline containing phosphate buffer (0.02 M, pH 7.2). The strains and mouse passages of dengue virus serotypes used in the experiments were dengue type 1 (strain Hawaii, passage 74), dengue type 2 (strain New Guinea B, passage 54), dengue type 3 (strain H-87, passage 27), dengue type 4 (strain H-241, passage 13), dengue type 5 (strain Th-36, passage 16), and dengue type 6 (strain Th-Sman, passage 15).

Cell cultures. Primary rhesus monkey kidney cell monolayer cultures were prepared and grown in 3-oz flint-glass prescription bottles by the method of Hsiung and Melnick (7). Established lines of high-passage (>100) rhesus monkey kidney [LLC-MK2 (4)] were maintained as Roux stock cultures, using Eagles basal medium (2), with Hanks salts (Earles salts for maintenance medium) supplemented with antibiotics (100 µg of penicillin per ml, streptomycin, and fungizone), 10% freshly harvested, unheated 4- and 8-week-old calf serum (2% for maintenance medium), glutamine (1 ml of 200 mM per liter), and 10.0 ml of 7.5% filtered sodium bicarbonate per liter (30.0 ml/liter for maintenance medium).

LLC-MK2 stock was split 1:4 every 7 to 10 days for stock maintenance. Between days 3 and 5, 2 to 3 ml of 7.5% sodium bicarbonate was added to adjust pH to neutrality. In preparing 3-oz flint-glass prescription bottles, 6 LLC-MK2 Roux stock cultures were em-
ployed to make 160 LLC-MK₂ plaque bottles. In the trypsinization procedure, 2.0 ml of 2.5% trypsin was pipetted onto the cell-free, warm, inner surface of the medium-free Roux bottle cell monolayers, and the bottles were inverted to uniformly wet the cell monolayer. The monolayer was freed of the trypsin by inversion of the bottle, and digestion was allowed to proceed (about 5 min, depending on the age of the culture). Once the cell sheet began to peel, the trypsin was decanted and an equal volume of serum-free growth medium (120.0 ml) was poured into the rubber-stoppered Roux stock bottles. The digested cells were then shaken off and decanted into the cell dispensing flask.

Serum and other medium components were added to the dispensing flask in which the cells were being agitated constantly by a mixing bar. Cells were dispensed with a 10.0-ml Cornwall syringe. It is important to have the cells uniformly dispersed while dispensing them into the 3-oz bottles.

Mice. Suckling ICR mice from a colony maintained in the Yale Arbovirus Research Unit facilities were used for preparation of virus source.

Virus assay. The procedure of virus inoculation and preparation of nutrient agar overlay was reported previously, 163, (17).

RESULTS AND DISCUSSION

Inoculum size and time of adsorption. It has been common practice in the plaque assay of virus infectivity to use an inoculum size sufficient to cover the cell sheet. A variance of 0.1 to 0.5 ml of inoculum has been recommended. Rhesus kidney monolayers that received 0.1, 0.2, or 0.4 ml of inocula were incubated for 1, 2, or 4 hr and then overlayed without washing off the inoculum (Table 1). The highest titer was obtained by the use of the largest inoculum (0.4 ml), but no increase in titer resulted from the prolonged period of adsorption.

Adsorption time and temperature. When adsorption was allowed to occur at 5, 25, or 37 C, at three time periods (1, 2, and 4 hr), a loss of 1 log in virus titer was observed with dengue type 2 (New Guinea B strain) at 5 C on primary rhesus kidney cell monolayers. No significant difference in titers (4.9 to 5.2 log₁₀ plaque-forming units per 0.2 ml) was seen when the virus was adsorbed at 25 or 37 C. No evidence of an increase in virus titer was seen when the incubation time was extended from 1 to 2 or 4 hr.

Types of nutrient overlay. When dengue type 2 (New Guinea B strain) was assayed for infectivity in primary rhesus kidney cells with two types of nutrient overlay, namely, a high Mg²⁺ (0.1015 g/ml) and a low Ca²⁺ (0.0002 g/ml), and the regular Hsiung and Melnick overlay (7), it was found that the high-Mg²⁺ low-Ca²⁺ type showed a 0.6-log higher titer. This effect was repeatable in three additional experiments.

| Inoculum size (ml) | Titers at intervals between inoculation and overlay of a |
|--------------------|-----------------------------------------------|
| 0.1                | 4.9   | 4.5   | 4.9   |
| 0.2                | 5.3   | 5.3   | 5.3   |
| 0.4                | 5.5   | 5.6   | 5.7   |

*a Values are expressed as log₁₀ plaque-forming units per 0.2 ml.

Concentration of serum in the overlay medium. Doubling the serum concentration in the nutrient overlay on LLC-MK₂ cells had little effect in modifying the titer of the dengue virus types 1, 2, 3, and 4.

Sera: inactivated versus unheated. When three age classes of calf serum (newborn gamma, 4- and 8-week-old) were compared for dengue virus plaque production (Table 2), it was found that all three classes of unheated calf sera gave comparable titers. However, use of heat-inactivated 4-week-old calf serum allowed a 1.4-log greater plaque titer with dengue type 3 when compared to the plaque titer obtained with the unheated 4-week-old calf serum. Neither age nor inactivation of the serum modified the experimental dengue plaque appearance time or size. Dengue type 1 plaques appeared at 9 days and dengue types 2 to 6 appeared at 6 days. Dengue types 1, 2, 5, and 6 measured 0.5 mm in diameter and dengue types 3 and 4 measured 1.0 mm.

Replacement of serum protein in the nutrient agar overlay. No differences were seen in the plaque titers of any of six dengue serotypes tested in LLC-MK₂ monolayers when either normal mouse ascitic fluid or bovine plasma albumin (7.5%) was substituted for calf serum in the nutrient agar overlay.

Sodium bicarbonate concentration. Sodium bicarbonate was tested for its plaquing efficiency, in various concentrations ranging from 0.56 mg/ml of overlay to 8.8 mg/ml of overlay, with all dengue strains on LLC-MK₂ cells (Table 3). The lowest concentration (0.56 mg/ml) delayed all strains except dengue type 4 virus. The highest concentration (8.8 mg/ml) was toxic for the LLC-MK₂ cells. Bicarbonate concentrations of 0.56 to 4.4 mg/ml gave comparable titers with all dengue viruses, with the greatest variation (in plaque counts) occurring with dengue types 4 and 6. The concentration of sodium bicarbonate was important for dengue plaque size modification,
especially for dengue types 2, 3, and 4, in which two- to eightfold differences in plaque sizes were noted.

Neutral red: effect of concentration and sterilization. The normal neutral red concentration (0.00166 g%) which gives the best color contrast was increased from 0.00166 to 0.00332 g% (Table 4).

This two-fold increase in neutral red concentration reduced the virus titer as much as 2.2 logs with type 2 dengue virus. When the neutral red concentration was increased to 0.00664 g%, the cell sheet degenerated before plaques appeared. No great differences in virus titers were observed when filtered or autoclaved (121.2 C for 15 min) neutral red was used for plaquing the dengue virus strains.

Effect of overlay pH. Titer, plaque size, and development times of dengue virus types 1, 2, and 4 were relatively unaffected at pH values ranging from 6.6 to 8.6 (Table 5). With dengue virus type 3, the titer decreased from 6.8 to 6.0 log₁₀ plaque-forming units per 0.2 ml at pH 8.6. The plaque size also decreased from 3 to 4 mm to 2 mm at pH 8.6. With dengue types 5 and 6, the plaque development times were increased from 7 to 10 days.

Cell aging. Two lots of LLC-MK₂ cells, 3 and 24 days old, respectively, were employed to assay

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**Table 2. Effect of age and heat on calf sera used for plaquing dengue virus in LLC-MK₂**

| Virus type | Fetal agamma | 4 Weeks | 8 Weeks |
|------------|--------------|---------|---------|
|            | Inactivated  | Unheated| Inactivated | Unheated | Inactivated | Unheated |
| Titer<sup>a</sup> | Plaque Size | Time | Plaque Size | Time | Plaque Size | Time | Plaque Size | Time | Plaque Size | Time |
| mm | days | mm | days | mm | days | mm | days | mm | days | mm | days |
| 1 | 5.4 | 0.5 | 9 | 5.1 | 0.5 | 9 | 5.7 | 0.5 | 9 | 5.4 | 0.5 | 9 |
| 2 | 6.9 | 0.5 | 6 | 6.7 | 0.5 | 6 | 7.2 | 0.5 | 6 | 6.8 | 0.5 | 6 |
| 3 | 6.2 | 1.0 | 6 | 6.1 | 1.0 | 6 | 7.6 | 1.0 | 6 | 6.2 | 1.0 | 6 |
| 4 | 6.3 | 1.0 | 6 | 6.0 | 1.0 | 6 | 6.4 | 1.0 | 6 | 6.0 | 1.0 | 6 |
| 5 | 6.1 | 0.5 | 6 | 6.1 | 0.5 | 6 | 6.1 | 0.5 | 6 | 6.1 | 0.5 | 6 |
| 6 | 4.6 | 0.5 | 6 | 4.5 | 0.5 | 6 | 5.1 | 0.5 | 6 | 4.7 | 0.5 | 6 |

<sup>a</sup> Inactivation at 56 C for 30 min.
<sup>b</sup> Hydrated Gibco.
<sup>c</sup> Processed in this laboratory (lot 13).
<sup>d</sup> Gibco lot 61312G.
<sup>e</sup> Values are expressed as log₁₀ plaque-forming units per 0.2 ml.

**Table 3. Effect of varying the sodium bicarbonate concentration on dengue virus plaque formation in LLC-MK₂**

| Virus type | 0.56 | 1.1 | 2.2 | 4.4 |
|------------|------|-----|-----|-----|
| Titer<sup>a</sup> | Plaque development | Titer | Plaque development | Titer | Plaque development | Titer | Plaque development | Titer |
| mm | days | mm | days | mm | days | mm | days | mm | days |
| 1 | — | — | — | — | — | — | — | — | — |
| 2 | 7.3 | 1 | 12 | 7.2 | 3 | 6 | 7.2 | 2 | 6 |
| 3 | 6.2 | 1 | 12 | 6.4 | 3 | 6 | 6.6 | 3 | 6 |
| 4 | 6.2 | 0.5 | 6 | 6.1 | 3 | 6 | 6.4 | 4 | 6 |
| 5 | 5.9 | 1 | 12 | 6.2 | 1 | 6 | 6.1 | 0.5 | 6 |
| 6 | 5.3 | 1 | 12 | 5.9 | 2 | 6 | 5.1 | 2 | 6 |

<sup>a</sup> Values are expressed as log₁₀ plaque-forming units per 0.2 ml.
<sup>b</sup> Sheet degenerated.
TABLE 4. Effect of various neutral red concentrations and method of sterilization on dengue virus plaque formation in LLC-MK₂

| Virus type | Neutral red concn | Type of sterilization |
|------------|-------------------|-----------------------|
|            | 0.00166 g%       | 0.00332 g%           | Filtered | Autoclaved |
|            | Plaque development | Titer | Plaque development | Titer | Plaque development |
|            | Size | Time | Size | Time | Size | Time | Size | Time | Size | Time |
| 1          | 5.7  | 1    | 11   |       | 5.7  | 0.5  | 8    |       |       |       |       |       |
| 2          | 7.2  | 0.5  | 6    |       | 7.2  | 0.5  | 6    |       |       |       |       |       |
| 3          | 6.7  | 1    | 6    |       | 6.7  | 1    | 6    |       |       |       |       |       |
| 4          | 6.4  | 1    | 6    |       | 6.4  | 0.5  | 6    |       |       |       |       |       |
| 5          | 6.1  | 0.5  | 6    |       | 6.1  | 0.5  | 6    |       |       |       |       |       |
| 6          | 5.1  | 0.5  | 6    |       | 5.1  | 0.5  | 6    |       |       |       |       |       |

* Values are expressed as log₁₀ plaque-forming units per 0.2 ml.
* Cells degenerated.

TABLE 5. Effect of pH of the agar-overlay in plaquing dengue virus in LLC-MK₂ cells

| Virus type | pH of overlay |
|------------|--------------|
|            | 6.6 | 7.1 | 8.2 | 8.4 | 8.6 |
|            | Plaque development | Titer | Plaque development | Titer | Plaque development | Titer | Plaque development | Titer |
|            | Size | Time | Size | Time | Size | Time | Size | Time | Size | Time |
| 1          | 5.9  | 1    | 10   |       | 5.8  | 1    | 10   |       | 5.5  | 1    | 10   |       |
| 2          | 7.1  | 1-2  | 10   |       | 7.3  | 1-2  | 7    |       | 6.9  | 1-2  | 7    |       |
| 3          | 6.8  | 3-4  | 7    |       | 6.6  | 3-4  | 7    |       | 6.3  | 3-4  | 7    |       |
| 4          | 6.4  | 3-4  | 7    |       | 6.1  | 3-4  | 7    |       | 6.2  | 4-6  | 7    |       |
| 5          | 6.7  | 1-2  | 7    |       | 6.6  | 1-2  | 7    |       | 6.4  | 1-2  | 7    |       |
| 6          | 5.6  | 1-2  | 7    |       | 5.5  | 1-2  | 7    |       | 5.3  | 1-2  | 7    |       |

* pH was adjusted with 1 N HCl or 1 N NaOH after bicarbonate addition.
* Values are expressed as log₁₀ plaque-forming units per 0.2 ml.

all six dengue type strains. There was little difference in virus plaquing titers.

Whereas the data showed that higher dengue type 2 titers were obtained by the use of the largest inoculum (0.4 ml) for cell adsorption purposes, the difference in titer was small and the practical use of 0.1 to 0.2 ml of inoculum for 1 hr was considered satisfactory. Hopkins et al. (5) reported that the plating efficiency of African horse sickness virus in monkey kidney cells was greater when 0.1 ml of inoculum was used rather than 0.2, 0.3, 0.4, or 0.5 ml and that the titer per milliliter decreased as the volume of inoculum was increased.

Adsorption at 5 C reduced plaque efficiency (1 log) of dengue type 2 (New Guinea B strain) on primary rhesus kidney cell monolayers, but no great difference in titers was found by adsorption either at 25 or 37 C. An increased plaque titer with dengue type 2 did not occur when the adsorption time was doubled, whereas Hotta et al. (6) found that the titer of type 1 (Mochizuki strain) was increased with more adsorption time. The adsorption time for other reported arboviruses has varied from 1.5 to 4 hr (5, 9-11, 12). In practice, arbovirus cell adsorption should be limited to 2 hr to avoid dehydration of the cell sheet.

Although a 37 C adsorption temperature was normally selected as physiological and in some cases necessary to attain highest efficiency of adsorption with Japanese B encephalitis virus (12), adsorption of dengue 2 was optimal at 25 C as was Colorado tick fever virus (1).
In an earlier publication, a modified Hsiung-Melnick overlay was recommended for plaquing dengue viruses (3). The presence of arginine and oxalacetate was not necessary for optimal plaquing conditions, and the experiments reported here were done omitting these two compounds from the overlay medium. The increased magnesium and decreased calcium ion concentration-modification did, however, allow dengue 2 virus to increase its titer by 0.6 log.

Heat-inactivated (30 min at 56°C) calf, horse, and chicken sera at various concentrations, alone or in several combinations, were incorporated into the methyl cellulose and secondary agar overlays for dengue 2 plaquing (15). Although plaquing efficiency was not significantly affected by any of these modifications tried, the plaques were larger with horse serum. During this study, we processed our own calf serum from 4-week-old calves and compared three age classes of calf sera for plaque efficiency; the three age classes of unheated calf sera gave comparable titers.

Although no differences in plaque titers resulted in the LLC-MK2 cell system when calf serum was replaced with mouse ascitic fluid in the nutrient agar overlay, this same procedure was shown to reduce or abolish the plaque titer for dengue type 6 in the primary rhesus cell system.

We found it practical to incorporate neutral red (3.0 ml of 1:1000 concentration per 180 ml—final concentration 0.00166 g%) overlay in the agar at the time of overlaying to follow plaque development and as an aid in eliminating the manipulations of a second agar overlay. However, it cannot be said that a single agar overlay promotes higher dengue plaque quantitation than those titers obtained by use of a double agar overlay, in which neutral red is added to the second agar overlay; these experiments were not performed.

A twofold increase in the normal amount of neutral red (0.0032 g%) reduced the virus titer as much as 2.2 logs with dengue type 2 virus, with complete cell destruction occurring, when the neutral red concentration was increased fourfold (0.0064 g%). Neutral red concentration of 0.003 and 0.06 g% likewise significantly affected plaque number or size of Japanese B encephalitis virus in chick embryo cell under agar (11).

No advantage was found in using filtered neutral red as opposed to autoclaved neutral red for maintaining all dengue virus titers at optimal concentration in LLC-MK2 cells.

Dengue virus plaquing in the LLC-MK2 cell system gave good results over a wide pH range (pH 6.6 to 8.6). Dengue type 3 titer and plaque size decreased at pH 8.6, whereas the plaque development times of dengue types 5 and 6 were increased at this same pH. It has been found by others (1) that Colorado tick fever virus plaque production was relatively insensitive to a variation in pH between 7.1 and 8.1 and plaque formation failed to occur at pH 7.0 or lower. When plaquing Japanese B encephalitis virus in hamster lung cells, an overlay medium pH under 6.6 or above 7.8 was not compatible with plaque production (8).

Cell (LLC-MK2) age apparently did not play a role in the assay plaque efficiency of the six dengue serotypes. Cell age, however, did play a role in plaque efficiency with two unrelated viruses, African horse sickness and Kunjin virus. Hopkins, et al. (5) inoculated Monkey kidney cell monolayers 3 days after their preparation with two strains of African horse sickness virus and obtained 1.0- to 1.5-mm plaques. However, cultures inoculated 7 days after their preparation showed plaques too small to measure.

Westway (19) found that the lower sensitivity of pig kidney cells in plaque assays for Kunjin virus was due to an aging effect on the cell monolayers.

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