Dengue Virus Plaque Development in Simian Cell Systems

II. Agar Variables and Effect of Chemical Additives

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Dengue type 2 virus, strain New Guinea B, plaqued with equal facility and titer under overlays containing six different grades of commercial agar in the LLC-MK₂ cell system. Doubling the agar volume on LLC-MK₂ cell monolayers increased the plaque development time of dengue type 1, strain Hawaii. Storage of agar at 56°C reduced or totally abolished dengue type 4, strain H-241, plaque titer in LLC-MK₂ cells. The influence of six known virus plaque-enhancing compounds on plaque development of all dengue virus serotypes was studied in two continuous simian kidney cell lines, LLC-MK₂ and Vero. In the absence of any chemical additive, plaque development of all dengue serotypes was more rapid (4 to 10 days) in the LLC-MK₂ line than in the Vero line (6 to 13 days). Increased plaque development time of type 1, strain Hawaii, by pancreatin and plaque-size doubling of dengue types 1 and 4 was the only advantage conferred by the addition of six chemical additives in the LLC-MK₂ cell system. Dengue types 1 and 6 failed to plaque in the Vero cell system unless aided by a plaque-enhancing compound; plagues of dengue types 2, 3, 4, and 5 appeared sooner (2 days) and were increased in plaque diameter. The optimal DEAE concentration for plaquing dengue type 1, strain Hawaii, was 100 μg/ml; plaque development either failed at lower concentrations or was inhibited at higher (200 μg/ml) concentrations.

The earliest dengue plaquing experiments (8–10; T. M. Stevens et al., Bacteriol. Proc., p. 132, 1962) revealed that agar contained certain substances which inhibit dengue virus plaque development. Since one of the inhibitors has been characterized as a sulfate polysaccharide (14), attention has been directed toward the use of more highly purified agar and utilization of chemical additives that reduce or neutralize the agar inhibitor.

This investigation was undertaken to measure the effect of purified commercial agars and various chemical additives on dengue virus plaque development in two different continuous simian kidney cell lines.

MATERIALS AND METHODS

Viruses. Wet-frozen (−70°C) virus stocks, prepared as 10% infant mouse brain suspensions in a 7.5% solution of bovine plasma albumin in physiologica saline containing phosphate buffer (0.02 M, pH 7.2) were used in all experiments.

Cell cultures. Established cell lines of high passage (>100) Vero green monkey kidney (17) and LLC-MK₂ rhesus monkey kidney (6) were maintained in 32-oz bottles, and 3-oz bottle cultures were prepared according to methods described in the preceding paper (11).

Mice. Swiss mice (CD-1; 1 to 2 days old) from a colony maintained in the Yale Arbovirus Research Unit were used for the preparation of virus stock.

Virus assay. Procedures of virus inoculation and preparation of nutrient agar overlay were reported previously (4, 12). Virus dilutions for titrations were made in Hanks saline containing 0.5% lactalbumin hydrolysate. The fluid medium was removed from the bottle cultures and 0.2 ml of each virus dilution was adsorbed for 1 hr at 37°C, after which 10 to 12 ml of a freshly prepared mixture of nutrient medium and agar was added. The nutrient overlay was prepared as follows. Modified 10× Earles solution was composed of solution A and solution B. For solution A, we combined 203.0 g of MgCl₂·6H₂O, 4.0 g of CaCl₂, 4.0 g of C₆H₈O₇, and water to a total volume of 800 ml. For solution B, we combined 272.0 g of NaCl, 6.0 g of KCl, 8.0 g of MgSO₄·7H₂O, 5.0 g of NaH₂PO₄·H₂O, 40.0 g of glucose, and water to a total volume of 3,200 ml. Solutions A and B were autoclaved separately, and it was important that A be added to B.

For the nutrient portion of the overlay, we combined 18.0 ml of modified 10× Earles solution, 57.1 ml of water (sterile, distilled), 3.6 ml of calf serum
(unheated), 0.2 ml of neutral red (1:100 filtered), 5.4 ml of NaHCO₃ (7.5%), 2.0 ml of antimicrobial stock (10,000 units of penicillin G per ml and 10,000 µg of streptomycin sulfate per ml), and diethylaminoethyl Dextran (Pharmacia, Uppsala, Sweden), pH 8.2.

For the agar (Ionagar no. 2; Colab Laboratories, Inc., Chicago Heights, III.) portion of overlay, the agar was prepared as required as a 3% (w/v) solution in 90.0-ml portions. The agar was sterilized by autoclaving for 15 min at 120 C, cooled to 56 C, and kept at that temperature until used.

The final overlay medium was then prepared by mixing the nutrient portion (90.0 ml) of the medium (kept at room temperature) with an equal volume of 3% agar. This mixture was poured onto the cell-free portion of the culture bottle (to reduce the temperature further) before allowing contact of the overlay medium with the cell sheet.

The bottles were stoppered, placed cell-sheet down at room temperature, and immediately covered with aluminum foil. After hardening of the agar, the bottles were inverted, incubated in the dark at 37 C, and examined daily for the appearance of plaques.

RESULTS

Agar types. Commercial grades of agar from five manufacturers (Table 1) did not significantly diminish nor increase the dengue type 2 (New Guinea B strain) titer on LLC-MK₂ cells; all agar lots tested gave titers ranging between 6.4 and 6.8 log₁₀ plaque-forming units per 0.2 ml. Plaque size was diminished (1.0 mm in diameter) with agar (Difco) and was largest with Ionagar and French agarose. With respect to plaque clarity, Noble, Behringwerke, and English agarose preparations supported clear plaque development; Ionagar and French agarose gave the next best clarity. Plaque clarity was acceptable in agar (Difco) but was the poorest tested. Substitution of agar (Difco) for the purified agars in the nutrient agar overlay adversely affected dengue virus plaquing in the Vero cell line, despite the presence of diethylaminoethyl (DEAE); the unpurified agar completely suppressed dengue types 1, 5, and 6 plaques and delayed (2 to 3 days) the plaque emergence of dengue types 3 and 4.

### Table 1. Effect of agar type (purity and manufacturer) on dengue type 2, strain New Guinea B, in LLC-MK₂ cells

| Agar type          | Virus titer | Plaque diam (mm) at days |
|--------------------|-------------|-------------------------|
|                    |             | 9 | 11 | 15 | 18 |
| Agar               |             | 6.4 | 1.0 | 1.0 | 1.0 |
| Special agar       |             | 6.8 | 1.0 | 1.0 | 1.0 |
| Nobel agar         |             | 6.6 | 1.0 | 1.0 | 1.0 |
| Pure agar, Behringwerke |     | 6.6 | 1.0 | 1.0 | 1.0 |
| Agarose            |             | 6.5 | 1.0 | 1.0 | 1.0 |
| Agarose            |             | 6.5 | 1.0 | 1.0 | 1.0 |

* Expressed as log₁₀ plaque-forming units per 0.2 ml.

### Table 2. Effect of doubling the Agar Overlay volume on dengue virus plaquing in LLC-MK₂ cells

| Dengue type | Strain   | Nutrient overlay volume | Plaque development | Plaque development |
|-------------|----------|-------------------------|--------------------|--------------------|
|             |          | Regular (10.0 ml)       | Double (20.0 ml)   |
|             |          | Size | Time | Titer | Size | Time |
|             |          | mm    | days |       | mm    | days |
| 1           | Hawaii   | 74    | 5.8  | 1-2   | 10    | 5.3  | 0.5-1 | 7   |
| 2           | Trini-dad| 54    | 7.3  | 1     | 7     | 7.0  | 1     | 7   |
| 3           | H-87     | 27    | 6.5  | 2-3   | 7     | 6.1  | 2     | 3   |
| 4           | H-241    | 13    | 6.0  | 2     | 7     | 5.9  | 2     | 7   |
| 5           | Th-36    | 16    | 6.4  | 1     | 7     | 6.0  | 1     | 7   |
| 6           | Th-Sman  | 15    | 5.4  | 1     | 7     | 5.4  | 1     | 7   |

* Ionagar no. 2.

† Expressed as log₁₀ plaque-forming units per 0.2 ml.

data...
TABLE 3. Effect of using old molten agar and diethylaminoethyl (DEAE) on dengue type 4 in LLC-MK₂ cells

| Amt of DEAE | Values after agar kept at 56 C for days¹ |
|-------------|------------------------------------------|
| None        | 6.2 5.8 5.8 4.9 5.1 CD CD CD           |
| 100 µg/ml   | 6.2 5.7 5.9 6.0 5.6 CD CD CD          |

¹ Values are expressed as log₁₀ plaque-forming units per 0.2 ml.
² Special Noble agar.
³ Cells degenerated.

prepared agar (Table 3). After storing the agar at 56 C for 5, 6, and 7 days, the cell sheets degenerated. After 4 days storage at 56 C and in the absence of DEAE, the virus titer fell from 6.2 to 5.1 log₁₀ plaque-forming units per 0.2 ml, whereas in the presence of DEAE the virus titer fell only from 6.2 to 5.6 log₁₀ plaque-forming units per 0.2 ml.

Toxicity of plaque-enhancing compounds in LLC-MK₂ and Vero cell monolayers. Six chemicals were tested for their toxicity in LLC-MK₂ and Vero monolayers (Table 4) at various concentrations in twofold increments. LLC-MK₂ cells were more resistant to the compounds tested than were the Vero cells, as monitored by cell monolayer survivals. Whereas 40 units of heparin were toxic to Vero cells, LLC-MK₂ cells were resistant to 80 units of the drug. Vero cells, which normally show monolayer survival times exceeding 30 days, survived a DEAE dose of 800 µg/ml for 14 days, but degenerated on the 16th day. Protamine sulfate in a dose of 0.4 µg/ml was toxic to LLC-MK₂ cells, whereas Vero cells were resistant to this dose.

Effect of six recognized plaque-potentiating chemical compounds on the plaque development of all dengue serotypes in LLC-MK₂ and Vero cell monolayers. Six chemical compounds were studied for their dengue plaque-enhancing or suppressing effects (Table 5). The concentrations used were based on toxicity level experiments carried out with LLC-MK₂ and Vero cells. Specific plaque development data of all dengue serotypes, in the absence of plaque-potentiating compounds for the LLC-MK₂ cell system, were presented in a previous paper (4).

In the absence of any plaque-enhancing additive, dengue plaque development was more rapid (4 to 10 days) in the LLC-MK₂ cell system than in the Vero cell system (8 to 13 days). Plaques appeared earlier and were of a greater diameter in the LLC-MK₂ system. Dengue virus types 1 and 6 were unable to plaque in the Vero cell system without additives.

Dengue type 1 plaques failed to develop in Vero cells unless DEAE, actinomycin, pancreatin, or glutamine were incorporated into the overlay. Plaque definition was best in the presence of glutamine. Dengue type 2 titred equally well in both the Vero and LLC-MK₂ cell systems, with a more rapid plaque appearance rate in the LLC-MK₂ line. Addition of DEAE or pancreatin to the nutrient overlay in the Vero cell monolayers doubled the size of the dengue type 2 plaques.

Whereas the appearance of dengue type 3 plaques required 4 to 6 days in LLC-MK₂ cells and ranged from 2 to 5 mm in size, 8 to 13 days were required for plaque development of this serotype in Vero cells. Addition of actinomycin or pancreatin to the Vero cell overlay increased plaque size from 1 to 2 to 4 mm.

Dengue type 4, strain H-241, showed rapid development of plaques in LLC-MK₂ cells but required 8 to 10 days in Vero cells and did not develop beyond 1 mm in diameter in Vero cells unless DEAE-dextran or actinomycin was added.

Dengue type 5 required 8 to 13 days to produce plaques in Vero cells but only 5 to 6 days in LLC-MK₂ cells. The largest plaque size occurred in the Vero cell line when DEAE-dextran was added to the overlay medium. Dengue type 6 uniformly plaked at 6 to 8 days in the LLC-MK₂ cell system with minor titer variations (5.6 to 6.1 log₁₀

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Table 4.

| Chemical          | Concentration tested per ml of overlay² | Toxic dose ³   |
|-------------------|----------------------------------------|----------------|
|                   | LLC-MK₂                                 | Vero          |
| Heparin           | 5 to 8 units                            | —²           |
| Pancreatin        | 0.025 to 4.0 µg                         | —             |
| Glutamine         | 0.5 to 8 mM ²                          | —             |
| Diethylaminoethyl | 50 to 800 µg                            | — 800        |
| Protamine sulfate | 0.1 to 1.6 µg                          | 0.4²         |
| Actinomycin       | 0.00125 to 0.05 µg                      | 0.025²       |

² Tested at twofold increasing concentrations; results at 16 days.
³ Toxic at 2 days.
⁴ Resistant to 800 µg at 14 days.
⁵ Resistant to 1.6 µg at 14 days.
⁶ Resistant to 0.05 µg at 14 days.
⁷ Resistant to 0.05 µg at 14 days.
TABLE 5. Effect of six recognized plaque-potentiating chemical compounds on plaque development of all dengue virus serotypes in LLC-MK$_2$ and Vero cell lines

| Chemical                  | First plaque appearance (days) | Plaque size at 13 days (mm) | Titer$^a$ |
|---------------------------|--------------------------------|-----------------------------|----------|
|                           | LLC-MK$_2$                     | Vero                        | LLC-MK$_2$ | Vero       | LLC-MK$_2$ | Vero       |
| 1, Hawaii$^b$             |                                |                             |          |
| None; control             | 10                             | 4                           | 1         | 6.3       | 10         | 4         | 1         | 6.3       |
| Heparin                   | 8                              | 4                           | 1         | 5.5       | 8          | 4         | 1         | 5.5       |
| Protamine sulfate         | 10                             | 1                           | 1         | 6.2       | 10         | 1         | 1         | 6.2       |
| DEAE-dextran              | 8                              | 13                          | 1         | 5.7       | 10         | 13        | 2         | 6.3       |
| Actinomycin               | 10                             | 13                          | 2         | 6.3       | 10         | 13        | 2         | 6.3       |
| Pancreatin                | 6                              | 13                          | 3         | 6.1       | 6          | 13        | 3         | 6.1       |
| Glutamine                 | 8                              | 13                          | 3         | 6.5       | 8          | 13        | 3         | 6.5       |
| 2, Tr-1751                |                                |                             |          |
| None, control             | 4                              | 8                           | 4         | 7.3       | 4          | 8         | 4         | 7.3       |
| Heparin                   | 4                              | 6                           | 2         | 7.5       | 4          | 6         | 2         | 7.5       |
| Protamine sulfate         | 6                              | 6                           | 1         | 7.6       | 6          | 6         | 1         | 7.6       |
| DEAE-dextran              | 5                              | 6                           | 2         | 7.6       | 5          | 6         | 2         | 7.6       |
| Actinomycin               | 5                              | 6                           | 4         | 7.5       | 5          | 6         | 4         | 7.5       |
| Pancreatin                | 4                              | 6                           | 1         | 7.3       | 4          | 6         | 1         | 7.3       |
| Glutamine                 | 5                              | 13                          | 2         | 7.3       | 5          | 13        | 2         | 7.3       |
| 3, H-87                   |                                |                             |          |
| None, control             | 5                              | 10                          | 5         | 6.4       | 5          | 10        | 5         | 6.4       |
| Heparin                   | 4                              | 10                          | 3         | 6.7       | 4          | 10        | 3         | 6.7       |
| Protamine sulfate         | 6                              | 13                          | 4         | 6.8       | 6          | 13        | 4         | 6.8       |
| DEAE-dextran              | 4                              | 8                           | 4         | 6.9       | 4          | 8         | 4         | 6.9       |
| Actinomycin               | 4                              | 8                           | 3         | 6.7       | 4          | 8         | 3         | 6.7       |
| Pancreatin                | 4                              | 8                           | 2         | 6.7       | 4          | 8         | 2         | 6.7       |
| Glutamine                 | 5                              | 13                          | 3         | 6.8       | 5          | 13        | 3         | 6.8       |
| 4, H-241                  |                                |                             |          |
| None, control             | 4                              | 10                          | 2         | 0.56      | 4          | 10        | 2         | 0.56      |
| Heparin                   | 4                              | 10                          | 2         | 1         | 4          | 10        | 2         | 1         |
| Protamine sulfate         | 4                              | 10                          | 4         | 1         | 4          | 10        | 4         | 1         |
| DEAE-dextran              | 4                              | 8                           | 3         | 5         | 4          | 8         | 3         | 5         |
| Actinomycin               | 4                              | 8                           | 4         | 6         | 4          | 8         | 4         | 6         |
| Pancreatin                | 4                              | 8                           | 2         | 6         | 4          | 8         | 2         | 6         |
| Glutamine                 | 4                              | 10                          | 2         | 1         | 4          | 10        | 2         | 1         |
| 5, Th-36                  |                                |                             |          |
| None, control             | 5                              | 10                          | 2         | 1         | 5          | 10        | 2         | 1         |
| Heparin                   | 6                              | 8                           | 0.51      | 6.9       | 7.1       | 8         | 0.51      | 6.9       |
| Protamine sulfate         | 6                              | 13                          | 2         | 1         | 6.8       | 6          | 13        | 2         | 6.8       |
| DEAE-dextran              | 5                              | 8                           | 1         | 4         | 6.7       | 6          | 8         | 1         | 6.7       |
| Actinomycin               | 5                              | 10                          | 2         | 2         | 6.9       | 6          | 10        | 2         | 6.9       |
| Pancreatin                | 6                              | 10                          | 1         | 1         | 6.7       | 6          | 10        | 1         | 6.7       |
| Glutamine                 | 6                              | 10                          | 2         | 1         | 5.9       | 6          | 10        | 2         | 5.9       |

$^a$ Expressed as $\log_{10}$ plaque-forming units per 0.2 ml.

$^b$ Dengue virus serotype and strain.

$^c$ Absence of plaques at lowest dilution tested.

$^d$ Plaques developed but not clear enough to count.

**DISCUSSION**

Although dengue virus plaquing was first accomplished by using methyl cellulose (2%) as the solidifying agent in the overlay (9), agar of various purities and concentrations (0.7 to 2.0%) has been preferred by most laboratories for dengue plaquing (4, 12, 13). Unpurified agar (Difco) was not acceptable for optimal dengue plaquing, nor
TABLE 6. Dengue virus plaque formation in Vero cells: effect of diethylaminoethyl (DEAE)* in the agar overlayb

| Dengue virus type and strain | None | 50 | 100 | 200 |
|-----------------------------|------|----|-----|-----|
|                             | Size | Titer | Size | Titer | Size | Titer | Size | Titer |
| 1, Hawaii                   | days | mm  | days | mm  | days | mm  | days | mm  |
| 2, Tr-1751                  | 7    | 2   | 5.8  | 7    | 2   | 5.7  | 7    | 2   | 5.8  | 7    | 2   | 5.9  |
| 3, H 87                     | 11   | 3   | 4.9  | 13   | 2   | 5.0  | 10   | 3   | 4.9  | 13   | 2   | 4.3  |
| 4, H 241                    | 13   | 2   | 5.3  | 13   | 3   | 5.7  | 13   | 2   | 5.3  | 13   | 5   | 5.5  |
| 5, Th-36                    | 7    | 2   | 5.9  | 8    | 2   | 5.6  | 8    | 2   | 5.7  | 8    | 3   | 5.7  |
| 6, Th-Sman                  | 9    | 2   | 4.2  | 13   | 3   | 4.2  | 15   | 2   | 4.0  | 13   | 2   | 4.3  |

* Pharmacia, Inc., New Market, N.J.
b Ionagar no. 2.

Plaque development after treatment with DEAE (μg/ml)

were all the grades of special Nobel agar. Substitution of Ionagar #2 for Difco agar and special Noble agar has given dengue plaques of good clarity, size, and development times comparable to the highly purified agaroses (12).

Doubling the volume of the nutrient agar overlay failed to modify the titer, plaque size, and development time for dengue types 2, 3, 4, 5, and 6. Dengue type 1 apparently differs in its oxygen requirement since plaque development is accelerated by reducing available oxygen (increasing the thickness of the overlay). This influence of oxygen has been demonstrated, on other arboviruses, wherein the Chikungunya and O’nyong-nyong viruses have been shown to be markedly affected by oxygen tension (2).

The common practice of preparing molten agar a number of days (1 to 4) prior to dengue plaque experiments is contraindicated by experiments presented here in which the highest titers were obtained with freshly prepared molten agar.

The cell spectra for plaquing dengue viruses has been extended in these studies by the use of the continuous-line Vero cells. Hotta et al. (5) demonstrated plaquing of dengue type 1 in a continuous line of green monkey cells. Data presented here show the use of the continuous-line Vero cells for plaquing all six dengue virus types without the requirement of methyl cellulose. Other additives were required, however, to permit the plaquing of dengue types 1 and 6 in Vero cells, but not necessarily for the other four dengue types.

There were distinct advantages in using the LLC-MK2 cell line over the Vero cell line for plaquing dengue viruses: (i) plaque-enhancing chemicals were not required for maximal plaque development; (ii) plaques appeared sooner, i.e., 4 to 10 days as compared to 6 to 13 days; and (iii) plaque definition was of better quality and plaque size was generally larger.

Five of the six chemical additives tested for their dengue plaque-enhancing effects in LLC-MK2 and Vero cell monolayers have been used previously as aids in general arbovirus plaquing. Although pancreatin has not been used in arbovirus plaquing procedures, the enzyme has been recommended (15) for plaquing reovirus. Two of these (DEAE and actinomycin) have been tested for their dengue plaque-potentiation effects in KB (T. M. Stevens et al., Bacteriol. Proc., p. 132, 1962), LLC-MK2 (13), and pig kidney (15) cells.

Incorporation of DEAE in the agar overlay medium was reported to improve plaque definition or titer of dengue type 2, or both, in pig kidney (15) and KB (T. M. Stevens et al., Bacteriol. Proc., p. 132, 1962) cells. Our data extend the findings of the effect of DEAE on plaquing dengue virus in LLC-MK2 (13) cells.

Reproducibility of dengue virus titers in pig kidney cells was improved and plaques appeared earlier in cultures overlaid with agar medium containing 0.02 μg of actinomycin per ml (16). Although actinomycin increased plaque size for dengue types 1 and 4, no other significant influence of actinomycin on dengue plaque development was seen in LLC-MK2 cells. Actinomycin, however, did modify dengue plaque development in the Vero cell line.

Heparin was suggested as a dengue plaque
modifier because of its similarity in chemical structure to DEAE (1). Heparin did allow a 1- to 2-day earlier plaque production time with dengue types 1 and 3 in LLC-MK₂ cells and promoted earlier (2 days) plaque development of types 2 and 5. Plaque size of types 2, 3, and 5 was repressed by heparin in the LLC-MK₂ cell system.

The use of protamine sulfate was found to increase Western equine encephalomyelitis virus plaque size (3). This polysaccharide sulfate inhibitor doubled dengue type 4 plaque size in the LLC-MK₂ cell system, but decreased dengue types 2, 3, and 6 plaque size in the same cell line. Although type 2 plaque appearance size was shortened in the Vero cell system, a delay was seen with dengue types 3 and 5. Plaque size in Vero cells or appearance time in LLC-MK₂ cells was not greatly influenced by this compound.

It was reported (7) that Japanese encephalitis virus plaque formation in HL (a continuous hamster lung cell culture) cells did not occur or was delayed until the 5th day when 2 mM glutamine was omitted from the overlay. If glutamine was not added to the dengue type 1 overlay in Vero cells, plaques either failed to appear or were delayed until the 15th day. The chemical additives tested aided dengue plaquing in both LLC-MK₂ and Vero cells, with better effect in Vero cells (Table 5). Dengue types 2, 5, and 6 plaque development was unaffected by the additives used in the LLC-MK₂ cell line, whereas plaque enhancement was seen for all dengue types in the Vero cell line. Whereas dengue type 1 was most susceptible to the action of the additives tested in the LLC-MK₂ cell line, this same serotype was least affected in the Vero cell line. DEAE, actinomycin, and pancreatin were the most active in potentiating both plaque appearance time and size.

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