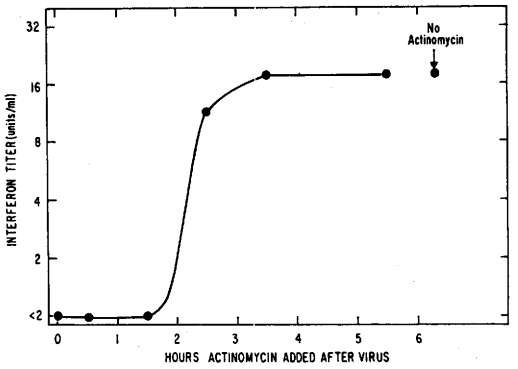

FIG. 1. Effect of addition of Actinomycin D, at different times, on production of interferon in CE cells infected with Chikungunya virus.

Monolayers of CE cells, in BME without serum, were exposed to Chikungunya virus at a multiplicity of 10. At indicated times after virus addition, actinomycin was added at a concentration of 0.2 μg/ml, and cultures allowed to incubate for a total of 6 hours after virus addition. Controls received no actinomycin.

plasm, then probably the messenger RNA is already in the cytoplasm by 1½ hours. If messenger RNA were being made continually almost from the time of addition of virus one would expect that the release from actinomycin control would be a more gradual process. The fact that an abrupt release from the control by actinomycin was found would indicate that messenger RNA for interferon became available not much before 1½ hours after infection. This is before much, if any, viral RNA has been made, but is well into the eclipse phase of the virus, where possibly other viral materials have been made. It should be mentioned that the present system was chosen because of the rapidity with which interferon is formed. What results would be found in a system where interferon is produced late during the course of infection remains to be determined.

Summary. Data have been presented which suggest that in the system of Chickungunya virus in chick embryo cells, the messenger RNA for interferon production is made available just shortly before 1½ hours after infection.

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Dengue Virus Plaque Formation in Rhesus Monkey Kidney Cultures.* (29851)

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Following Sabin's(1) adaptation of type 1 and type 2 Dengue viruses to mice, 2 and possibly 4 additional types have been isolated and identified by use of this experimental host system. Although several reports have presented evidence supporting the multiplication of mouse-adapted strains of Dengue viruses in a variety of tissue culture systems (2-9), cytopathic effects have been limited to rhesus monkey kidney(2-4), grivet monkey

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kidney(10), hamster kidney(6), HeLa(8), and KB(9) cell cultures but plaque formation limited to type 2 in KB cells(9). This report describes the use of a modified agar overlaid rhesus kidney cell system for performing plaque assays of the currently recognized Dengue virus types. As far as can be determined, this is the first description of a method using a universally susceptible system for plaquing all of the known Dengue virus serotypes.

Materials and methods. Viruses. All Dengue virus serotypes used in these studies were kindly supplied by Drs. W. McD. Hammon and Jordi Casals. The type, strain designation, and mouse passage level are listed in Table I. Virus stocks, stored at approximately —70°C, were prepared as 20% suspensions of infected mouse brains in a borate-saline buffer (pH 9.0) containing 5% bovalbumin.

Tissue cultures. Rhesus monkey kidney cell monolayer cultures were prepared and grown in 3-oz flint glass prescription bottles according to the method of Hsiung and Melnick(11). The overlay medium was prepared as follows:

A. Modified 10× Earle's solution

1. Solution A	
$MgCl_2 \cdot 6\ H_2O$	203.0 g
$CaCl_2$	4.0 "
Water *q.s.*	800.0 ml
2. Solution B	
NaCl	272.0 g
KCl	6.0 "
$MgSO_4 \cdot 7\ H_2O$	8.0 "
$NaH_2PO_4 \cdot H_2O$	5.0 "
Glucose	40.0 "
Water *q.s.*	3,200.0 ml

Solutions A and B were autoclaved separately, and it was important that A be added to B.

B. Nutrient portion of the overlay

Modified 10× Earle's solution	18.0 ml
Water (sterile distilled)	58.2 "
Calf serum	3.6 "
Neutral red (1:1000 filtered)	3.0 "
L-arginine (17.4 g per liter)*	1.0 "
$NaHCO_3$ (7.5%)	5.4 "
Antimicrobial stock†	0.2 "
Oxalacetic acid (10%)‡	0.6 "

* Stored at -20°C.

† Five million units of Penicillin G in 39.5 ml water added to 4.9 ml streptomycin sulfate at 0.5 g per ml.

‡ Stored at -20°C for not longer than 2 weeks.

C. Agar (Difco, Noble) 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 minutes at 120°C, cooled to 45°C, and kept at that temperature until used.

D. The final overlay medium was then prepared by mixing the nutrient portion (90.0 ml) of the medium with an equal volume of 3% agar.

Virus inoculation and overlay of RhMK cultures. The fluid medium was removed from the cultures, and 0.2 ml of each virus dilution adsorbed for 1-2 hours at 35°C, following which 10 to 12 ml of the freshly prepared mixture of nutrient medium and agar were added. The bottles were stoppered, placed cell sheet down at room temperature, and immediately covered with aluminum foil or brown paper to avoid photodynamic destruction of the cell sheet by the neutral red(12). After hardening of the agar the bottles were inverted, incubated in the dark at 35°C, and examined daily for the appearance of plaques.

Mouse inoculation. Infant mice, 1-2 days old, were inoculated intracerebrally with 0.02 ml of the virus dilution. LD_{50} titration endpoints were determined as described previously(13).

Tissue culture neutralization tests. Plaque reduction neutralization tests in rhesus monkey using kidney cultures were performed as reported previously(14). Type specific Dengue antisera, prepared in mice, were kindly supplied by Dr. Jordi Casals, The Rockefeller Foundation, New York City.

Results. Between the fifth and eleventh day after inoculation and overlay, small, distinct plaques were observed in cultures inoculated with each of the 6 Dengue virus types. Dilution endpoints varied depending upon the lot of rhesus kidney cells used and the apparent degree of contamination of certain lots with simian viruses. Generally, however, plaque titers in tissue culture were higher than LD_{50} endpoints in mice. Specificity of the plaques was ascertained by the use of plaque reduction neutralization tests using appropriate type-specific antisera.

A representative culture of each of the 6 Dengue virus types is illustrated in Fig. 1. The characteristics of the plaques, their times of appearance and size, and the titration ranges achieved in tissue culture and mice are summarized in Table I.

Discussion. Variations in agar overlay media have been reported to affect the development of plaques by several viruses. The findings reported here were the result of screening a large number of modifications based on previous reports concerning the

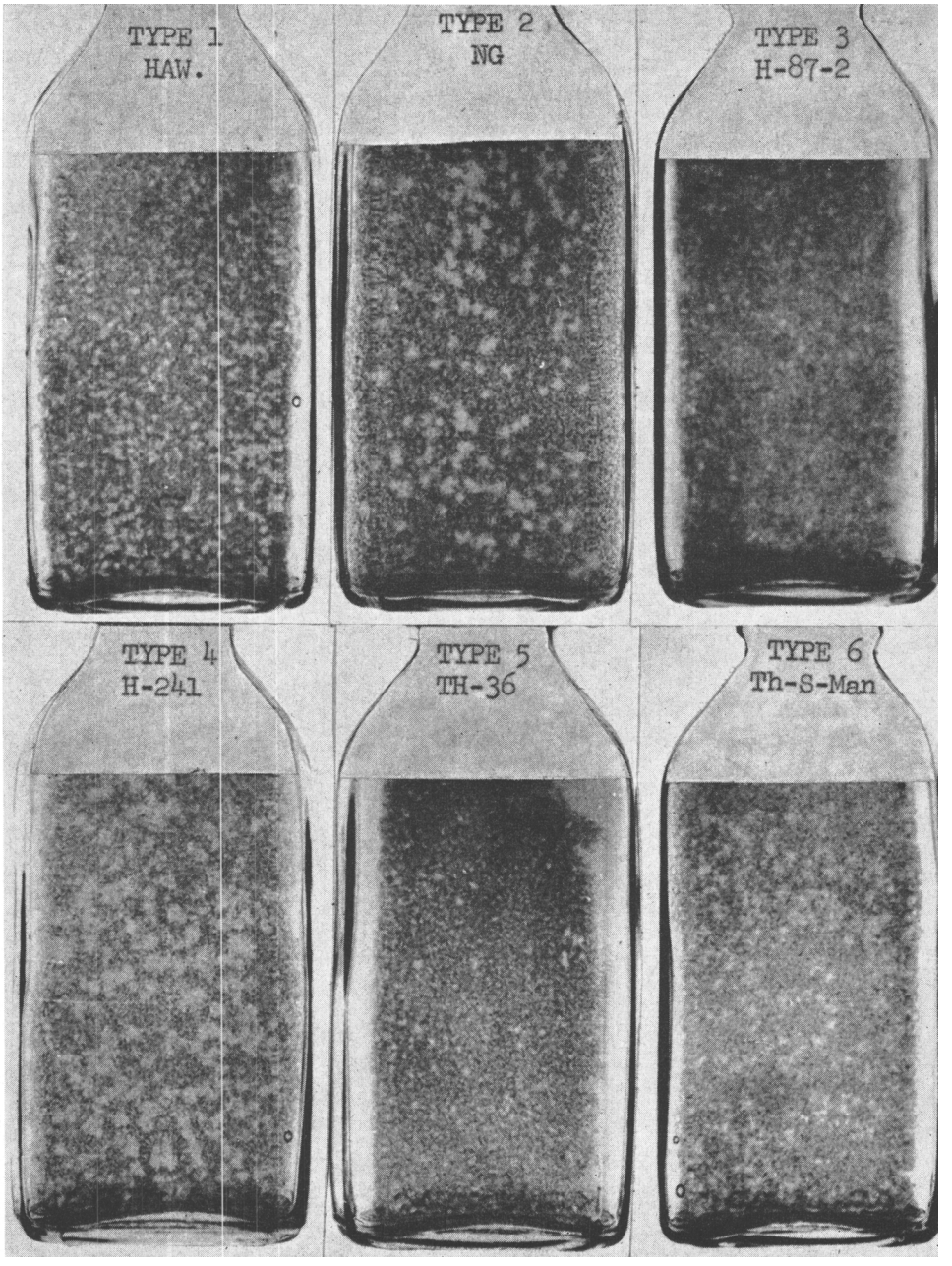

FIG. 1. Plaque formation by Dengue types 1-6 in rhesus monkey kidney bottle cultures, 10 days after inoculation.

TABLE I. Plaque Development, Titer Ranges in RhMK Tissue Cultures and Infant Mice, and Homologous RhMK Tissue Culture Neutralization Tests for Dengue Serotypes.

Dengue virus		Infant mouse	Plaque development		Tissue culture		
Serotype	strain	passage	Day	Size (mm diameter)	Log PFU reduction (homologous neutralization)	Log PFU titer range per 0.2 ml	Infant mouse LD_{50} per 0.02 ml
1	Hawaii	124	9–11	1	5.9	7.7–8.7	6.4
2	NG-B	3	5	1–2	4.5	3.8–5.5	5.0
3	H-87	23	7–9	1–2	5.9	4.2–5.3	4.9
4	H-241	24	7	1–3	4.8	4.8–6.5	4.0
5	TH-36	15	7–9	1–2	ND	4.8–8.6	5.7
6	TH-Sman	15	7	1–3	ND	5.3–8.5	4.0

ND = Not done.

effects of additional magnesium chloride(15), reduction of calcium chloride concentration (16), and arginine supplementation(17). Barile and Schimke(18) recently reported on the depletion of L-arginine by pleuropneumonia-like organisms as contaminants of tissue cultures. This effect may in part account for the essential nature of this ingredient for production of plaques in agar overlaid rhesus kidney cell cultures. It is also conceivable that the increased calcium ion concentration may block inactivation of virus by inhibitory substances in the agar(19).

The recognition during the past decade of the broad spectrum of clinical illnesses associated with Dengue viruses in different geographic areas and among different population groups even within a given area and the difficulties previously encountered in both virus isolation attempts and serologic methods, have pointed to the need for improved techniques in Dengue field studies. Although the system described here has not yet been tested adequately with field specimens, such studies are in progress.

Summary. Plaques were produced by mouse-adapted strains of the 6 recognized Dengue serotypes using a modified overlay of cultures of rhesus monkey kidney cells. Overlay modifications included changes in the magnesium and calcium ion concentration and addition of L-arginine and oxalacetic acid. Plaques 1-3 mm in diameter appeared 5-11 days after inoculation of cultures. Endpoints were generally higher in rhesus kidney cultures during parallel titrations of the virus in tissue culture and mice.

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