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Further Studies on Multiplication of Dengue Viruses in Various Host Systems.* (31310)

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Tissue culture systems susceptible to dengue virus infection offer additional methods for studies of the biological properties and the antigenic and immunogenic characteristics of this group of viruses. Cytopathic effects (CPE) are produced by selected dengue strains in primary cultures of rhesus monkey kidney(1,2) and hamster kidney(3) and in serial passage cultures of KB(4,5), BHK-21 (6), HeLa(7), human skin(8), porcine kidney(9), and stable rhesus(9). Plaque formation by these viruses has been described for only very few culture systems, namely KB (4), BHK-21(10), and primary rhesus monkey kidney(11). This report further characterizes the multiplication of the known dengue serotypes in mice and in certain susceptible culture systems.

Methods. Viruses. The following dengue virus strains were used: Type 1 (Hawaii), 124 infant mouse passages; Type 2 (Trinidad), 57 mouse passages; Type 2 (NG-B), 3 mouse passages; Type 3 (H-87), 24 mouse passages; Type 4, (H-241), 24 mouse passages; and provisional Types 5 (TH-36), 15 mouse passages, and 6 (TH-S-man), 15 mouse passages. Virus stocks were prepared as 20% infected mouse brain in borate buffered saline, pH 9, with 0.75% bovalbumin.

Tissue cultures. Primary monkey kidney cell monolayers were prepared and grown in 3 oz flint glass prescription bottles according to the methods of Hsiung(12). Roux bottle stocks of the continuous lines of rhesus monkey kidney(13) and porcine kidney(14) were grown in Eagle's minimal essential medium (15) fortified with 5-10% fetal calf serum and antimicrobials. Tryptose phosphate broth (1%) was added to the growth medium of the porcine kidney line. Cells of stock cultures were dispersed using 0.7% pronase (16) or 0.125% trypsin in serum-free growth medium and were planted at a concentration of 2.0-5.0 $\times$ 10^5 cells per milliliter. Medium changes were made at 3-5 days using growth medium but decreasing the serum concentration to 1.0% and doubling the bicarbonate concentration. Cultures were used for virus inoculation and overlay between 5-12 days after seeding.

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TABLE I. Plaque Development Characteristics and Comparative Titrations of Dengue Viruses in Various Tissue Culture Systems.

Dengue serotype	Strain	Tissue culture system	Plaque development: Time (days)	Plaque development: Diameter (mm)	Plaque development: Titer or titer range* (log PFU/0.2 ml)
1	Hawaii	Primary RMK	9–11	1–2	4.8–6.3
		LLC-Mk_2	9–12	2	5.3
		PS-Y-15	11–13	1–2	2.6
2	Trinidad	Primary RMK	5– 7	2	5.4–6.4
		LLC-Mk_2	4– 6	2–3	6.6–8.2
		PS-Y-15	4– 5	1–2	5.3
	New Guinea B	Primary RMK	5– 7	1–2	4.2–7.3
		LLC-Mk_2	5– 7	2–4	4.4–6.5
		PS-Y-15	7	1–2	3.8
3	H-87	Primary RMK	7– 9	1–2	4.1
		LLC-Mk_2	9–12	2	5.7–6.1
		PS-Y-15	13	1–2	3.7
4	H-241	Primary RMK	7	1–3	6.5
		LLC-Mk_2	4– 5	2–3	3.2–6.2
		PS-Y-15	4– 5	1–2	4.8
5	TH-36	Primary RMK	7– 9	1–2	4.8–8.6
		LLC-Mk_2	7	1–2	7.0
		PS-Y-15	5– 7	1	4.7
6	TH-S-man	Primary RMK	7	1–3	4.0–8.6
		LLC-Mk_2	7	1–3	5.3–5.7
		PS-Y-15	5	1–2	3.6–4.0

* Titer ranges are based on 5-6 replicate titrations of the same source.

Virus assay. Stock virus dilutions for titrations were made in either Hanks' saline containing 0.5% lactalbumin hydrolysate or in buffered saline containing calf serum or bovine serum albumin. If virus dilutions were prepared in buffered saline containing no protein, infectivity was substantially decreased in 3-4 hours. Bottle cultures were inoculated with 0.2 ml virus dilution, the inoculum adsorbed for 2 hours at 37°C, then overlaid (11). All bottles were incubated in an inverted position (agar at top) at 35°C until plaques could be counted. Suckling mice (CD-1 strain of Ha/ICR) were inoculated with 0.02 ml virus intracerebrally for comparative LD_{50} determinations.

Results. Tissue culture susceptibility. Rhesus monkey kidney (LLC-Mk_2) and porcine kidney (PS-Y-15) cultures were found to be highly susceptible to infection by the dengue viruses. Comparative data on plaque titrations in primary rhesus monkey kidney cells and rhesus kidney (LLC-Mk_2) and porcine kidney (PS-Y-15) passage cell lines are shown in Table I. Titer endpoints were generally more reproducible and plaques were more easily counted in the stable lines than in primary cultures. The variability of titration endpoints in primary rhesus monkey kidney cultures was undoubtedly influenced by the periodic presence of interfering simian virus contaminants from one culture lot to another.

Examples of the type of dengue plaques developing in serial passage lines are shown in Fig. 1 and 2. Plaques, 1-3 mm in diameter, developed in 4-7 days for dengue types 2, 4, 5, and 6 and in 9-13 days for dengue types 1 and 3. The type of agar used in the overlay did not affect virus titers significantly. Also, the presence or absence of certain heavy metal cations in the overlay had no influence on plaque titers using the passage cell lines. The effect of these cations in the primary rhesus culture system, as previously reported(11), was undoubtedly related to inhibition of interfering simian agents(17).

Growth curves of dengue viruses in infant mice. For comparison of multiplication rates of dengue viruses in tissue culture and mice, an experiment was designed to measure infective virus concentrations in brains taken at

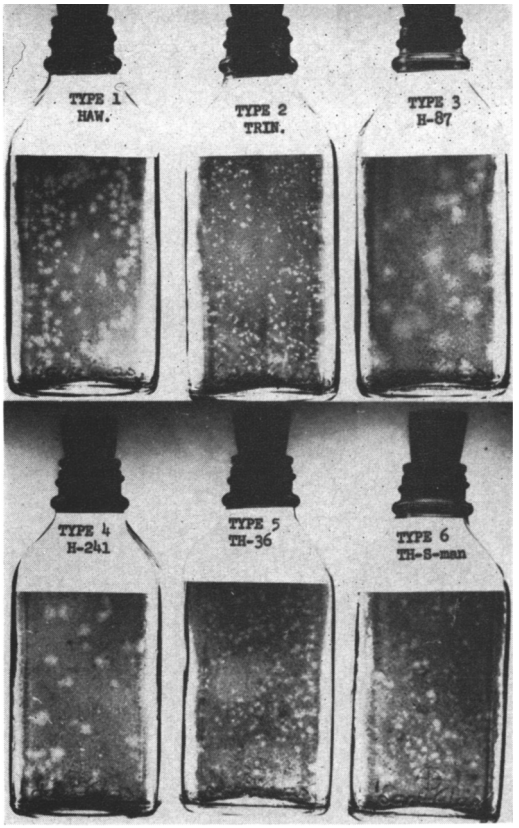

FIG. 1. Plaque formation by dengue viruses in rhesus monkey kidney (LLC-Mk_2) cell cultures.

24-hour intervals following infection. Titers were measured in rhesus monkey kidney cultures. Results of these comparisons are shown in Fig. 3. Logarithmic increases were seen with all dengue types which reached titers of 10^5 by the fourth day, except in the case of type 6, which reached a maximum titer 1 to 2 days later.

Adsorption, maturation, and release of dengue virus in various cell cultures. Dengue 2 (New Guinea B), a representative strain, was inoculated into primary rhesus kidney, LLC-Mk_2, and PS-Y-15 cell cultures at a concentration of 6.7-7.0 PFU (as determined by titration in LLC-Mk_2). Aliquots of infected cell culture fluids were removed and replaced with fresh media every 15 minutes. Intracellular or cell associated virus and extracellular (cell-free culture fluid) virus titers were determined on replicate series of cultures every 12 hours for 156 hours. Intracellular virus was defined as the infectious virus associated with cells following 3 washes and 3 freeze-thaw cycles. Results of the virus adsorption, maturation, and release experiments are shown in Fig. 4. About 99% of the virus was adsorbed onto the rhesus cells (primary and serial passage line) in 15 minutes. However, adsorption in the PS-Y-15 culture was poor, even after 120 minutes. Infective intracellular virus was recognizable in the passage lines about 24 hours prior to detection of extracellular virus in the maintenance fluids of corresponding sets of infected cultures. In the primary rhesus kidney cultures infected with dengue 2, neither intra- nor extracellular infectious virus could be detected after 156 hours. It appears that simian interfering agents restricted virus multplication in this instance.

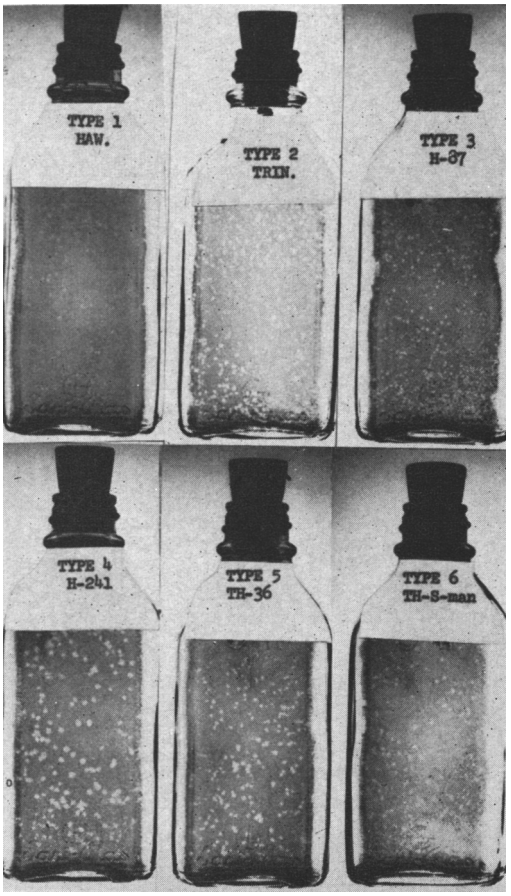

FIG. 2. Plaque formation by dengue viruses in porcine kidney (PS-Y-15) cell cultures.

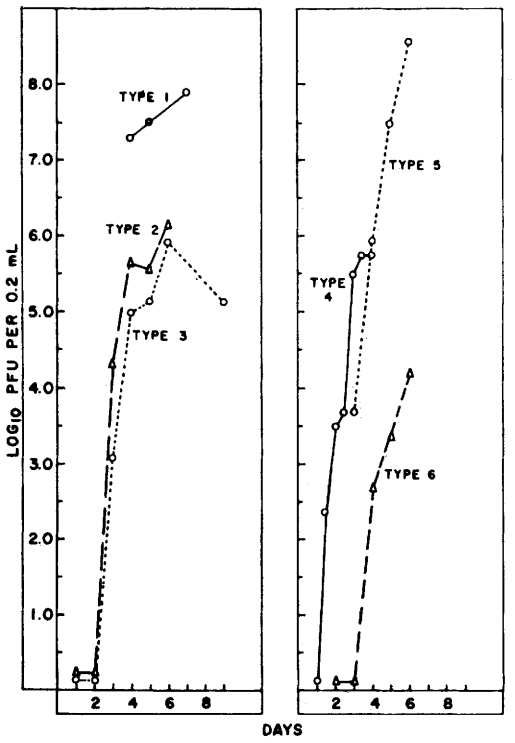

FIG. 3. Comparative growth curves of dengue viruses in infant mouse brain. Titers are determined in monkey kidney cultures.

Discussion. Cultures of primate origin appear to be the most satisfactory for studying the multiplication and plaque formation of dengue viruses *in vitro*. However, as much as 4 log variations do occur when viruses are titrated in primary cultures. This variation was also noted by Halstead(9) who found as much as 5 log differences with type 1 dengue by the interference assay method. Cultures of non-primate origin, such as porcine kidney, can be infected but are less efficient in adsorption of dengue virus inoculum and synthesis and release of new virus (Fig. 4). It appears that the infant mouse remains the most susceptible and sensitive host for propagation of these viruses (Fig. 3).

With the development of suitable plaque assay techniques for the dengue viruses, considerable versatility may be attained in performing neutralization tests as well as conducting studies on more basic aspects of the properties of these viruses. The wide variation in plaque forming unit endpoints obtained in primary cultures of rhesus monkey kidney has been obviated to some extent by utilizing the passage cell rhesus cultures (LLC-Mk_2), which are supposedly free of potential interfering simian agents. It has been common practice in the plaque assay of viral infectivity to use an inoculum size sufficient to cover the cell sheet. A variance of 0.1 to 0.4 ml inoculum has generally been recommended. The highest titer of New Guinea B using the same concentration of virus in various volumes was obtained by adsorbing an inoculum of 0.4 ml. No increase in the PFU titer resulted from prolonged adsorption of the virus inoculum, *i.e.*, longer than 1 hour. Adsorption of dengue viruses is usually efficient at either 25°C or 37°C, and similar titers are obtained at either temperature.

Time of appearance and rate of development of plaques were variable from one dengue serotype to another. Generally, 2 rates of plaque development in all culture systems used were recognized. Dengue types 2, 4, 5, and 6 produced plaques relatively soon, 4-7 days, while types 1 and 3 were characteristically slower in developing (Table I). Large and small plaque type variations were seen with types 1, 3, and 6 on LLC-Mk_2 cell cultures (Fig. 1). No such size variation was observed with the porcine kidney PS-Y-15 cultures (Fig. 2). The real function of the increased Mg^{++} and the decreased Ca^{++} in modified nutrient-overlay medium has not yet

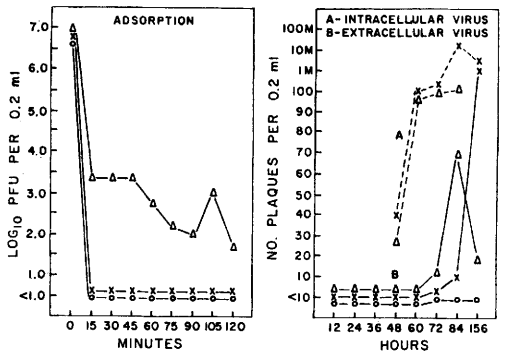

FIG. 4. Adsorption, multiplication, and release of dengue 2 in rhesus monkey kidney (primary and LLC-Mk_2 line) and porcine kidney (PS-Y-15) cell cultures. Key: X——X = LLC-Mk_2; O——O = primary rhesus; Δ——Δ = PS-Y-15.

been ascertained, since, at least with New Guinea B, use of the regular Hsiung-Melnick formula suffices. Furthermore, the lack of neither arginine nor oxalacetic appreciably affects New Guinea B virus plaquing efficiency; their inclusion, however, does aid in plaque clarity.

Summary. Host range susceptibility studies with mouse adapted dengue types 1-6 have shown that cultures prepared from kidney tissues of primate origin are more sensitive to dengue virus plaque formation than culture systems originating from tissues of non-primates, *e.g.*, porcine kidney. Plaques developed in cultures of rhesus monkey kidney (LLC-Mk_2 passage line) and porcine kidney (PS-Y-15 passage line) in 4-7 days for dengue types 2, 4, 5, and 6, and in 9-13 days for types 1 and 3. About 99% of dengue 2 was adsorbed onto primate kidney cultures in 15 minutes, but adsorption of virus in PS-Y-15 cultures was poor even after 120 minutes. When comparing multiplication rates of the dengue viruses in infant mice and two passage cell cultures, synthesis of new virus proceeded at about the same rate in both hosts. However, higher titers were usually evident in mouse brain preparations.

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Role of the Spleen in Interferon Production in Mice.* (31311)

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Heretofore, most experiments with interferon have utilized *in vitro* systems. Although *in vivo* interferon production has been previously reported(1,2), little is known about the site of interferon synthesis *in vivo*. Several investigators have demonstrated that leukocytes are capable of synthesizing this inhibitory substance(3,4), and it was therefore considered pertinent to determine to what extent leukopoietic organs are involved in the formation of interferon. The present report relates certain findings which indicate that the spleen plays a major role in interferon synthesis.

Materials and methods. Virus strains. The Cincinnati strain of Newcastle disease virus (NDV), obtained from Dr. Robert N. Chanock of the National Institutes of Health, and the Indiana strain of vesicular stomatitis virus (VSV), obtained from the American Type Culture Collection, were maintained by

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