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RESEARCH ON DENGUE IN TISSUE CULTURE.

II. Further Observations on Virus-Tissue Culture Affinity.[a, b]

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Yozo SHIMAZU, Hideo AOKI and Susumu HOTTA. *Research on Dengue in Tissue Culture.* II. *Further Observations on Virus-Tissue Culture Affinity.* Kobe J. Med. Sci. 12, 189–198, December 1966——Attempts were made to have dengue virus adapt to cultures of trypsinized chick embryo skin-muscle cells. Multiplication of the mouse-passaged type 1 Mochizuki strain dengue virus in this culture system was equivocal, showing only a prolonged maintenance of active virus. When the virus was successfully passed through *fuscatus* monkey kidney cell cultures, it proved to multiply significantly better in the chick embryo cultures, compared with the original mouse-passaged virus. In either case, however, serial transmission through the cell cultures was unsuccessful.

Both monkey kidney cell culture-passaged and mouse-passaged viruses of the Mochizuki strain multiplied well, exhibiting CPE, in cultures of an established African green monkey kidney cell line. In this system, "virus carrier state" or "persistent infection" took place. It was observed that the cellular degeneration was never total but that destruction and restoration of cellular sheets occurred intermittently and active virus was continually detected in the culture fluid for more than 400 days.

INTRODUCTION

Since 1955 a variety of tissue culture systems has been shown to support *in vitro* multiplication of dengue virus (Hotta, 1965),[8] and some of them are becoming useful tools for dengue experiments of various categories. However, tissue culture ranges of dengue virus are so far not wide, and even in dengue-susceptible culture systems, abundant growth of virus, accompanied by clear cytopathic effect, is not necessarily easy. This paper will deal with results of observations conducted with an aim to expand knowledge on so-called tissue culture spectrum of dengue virus. In performing the experiments, yellow fever virus was tested as a comparison. As is well known, dengue and yellow fever viruses, both belonging to the group B arboviruses, share certain important biologic features so that comparative studies on these two viruses would be of significance for understanding their characteristics.

MATERIALS AND METHODS

Virus.

Viruses employed were: dengue type 1 (D1), Mochizuki strain, of more than

a Aided by the Fund for Virus Research of Hyogo Prefecture.
b Preliminary descriptions of the work were presented at the 6th annual meeting of the Japan Society of Tropical Medicine, 22 November 1964, in Tokyo.
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170 mouse brain passages, and the same strain passed in *fuscatus* monkey kidney cell cultures for more than 20 generations; dengue type 2 (D2), Trinidad 1751 strain, of the 72nd mouse brain passage; and yellow fever (YF), 17D strain, of the 14th mouse brain passage, as well as its commercial vaccine.*

Cell Culture.

Cultures of trypsinized skin-muscle cells from 8 to 10 day-old chick embryos (CESM), and established kidney cell line cultures from the African green monkey (AGMK) **were used. Monolayers were grown in tubes, 15×2cm, or in 200ml dilution bottles. For morphological observations, cover slip cultures were prepared.

The following culture media were used:

Medium No. 1:

5% lactalbumin hydrolysate	10 parts
Bovine serum (inactivated)	10 parts
Hanks' solution (10-fold concentrated)	10 parts
Distilled water	70 parts

Medium No. 2:

5% lactalbumin hydrolysate	10 parts
Bovine serum ultrafiltrate (*Difco*)	20 parts
Hanks' solution (10-fold concentrated)	10 parts
Distilled water	60 parts

Medium No. 3:

YLE** (5-fold concentrated)	20 parts
Bovine serum (inactivated)	10 parts
Distilled water	70 parts

(** 0.1% yeast extract-0.5% lactalbumin hydrolysate in Earle's solution)

Medium No. 4:

YLE (5-fold concentrated)	20 parts
Bovine serum (inactivated)	5 parts
Distilled water	75 parts

Into all the media, penicillin and streptomycin were added at concentrations of 150u/ml and 50γ/ml, respectively. In general, Medium No. 1 was used for initial cultivation of CESM cells, Medium No. 2 for the same cultures after inoculation of the viruses, Medium No. 3 for the cultivation of AGMK cells, and Medium No. 4 for the AGMK cells after virus inoculation. Temperature for initial cellular cultivation was 37°C, and those following virus inoculation were either 37°C, 30°C 25 °C for CESM cultures, and 37 °C for AGMK cultures.

Techniques of preparation of cultures, inoculation and titration of virus, morphological examinations, *etc.*, were essentially the same as those described previously (Hotta, 1959).[2]

*Desiccated chick embryo vaccine, manufactured by the National Drug Co., U. S. A.

**Kindly supplied by Dr. I. Tagaya, of the National Institute of Health of Japan, who achieved the establishment of this cell line (Tagaya *et al*, 1962)[8]

RESULTS

Growth curve experiments.

Usually experiments were repeated two to four times in each virus-cell culture combination. There was no essential divergence in the results obtained, and typical examples are illustrated in Fig. 1 (regarding mouse-passaged viruses in CESM cultures), Fig. 2 (regarding TC-passaged viruses in CESM cultures), Fig. 3 (regarding mouse-passaged viruses in AGMK cultures) and Fig. 4 (regarding TC or chick embryo-passaged viruses in AGMK cultures).

The data indicate:

(1) Evidence for multiplication of mouse-passaged D1 virus in CESM cultures is equivocal. YF virus multiplies well under the same conditions, as has been universally confirmed.

(2) D1 virus passed in monkey kidney cultures shows apparently a little better growth in CESM cultures than does mouse-passaged virus of the same strain.

Serial passage of D1 virus through CESM cultures was attempted, starting with infected fluid from the tubes used for experiments recorded in Fig. 2. Data showed that mouse LD_{50}'s per 0.02 ml of the culture fluids were: $10^{1.5}$ in the second passage, $10^{1.5}$ in the third, $10^{1.5}$ in the fourth, $10^{1.5}$ in the fifth, and no virus in the fluid from the sixth passage. Similar passage experiments were repeated, obtaining essentially the same results.

(3) Dengue viruses, both mouse and TC-passaged, grow unequivocally in AGMK cultures. Similar pattern of growth is obtained with YF virus, either in the form of infected mouse brain homogenate or of commercial vaccine.

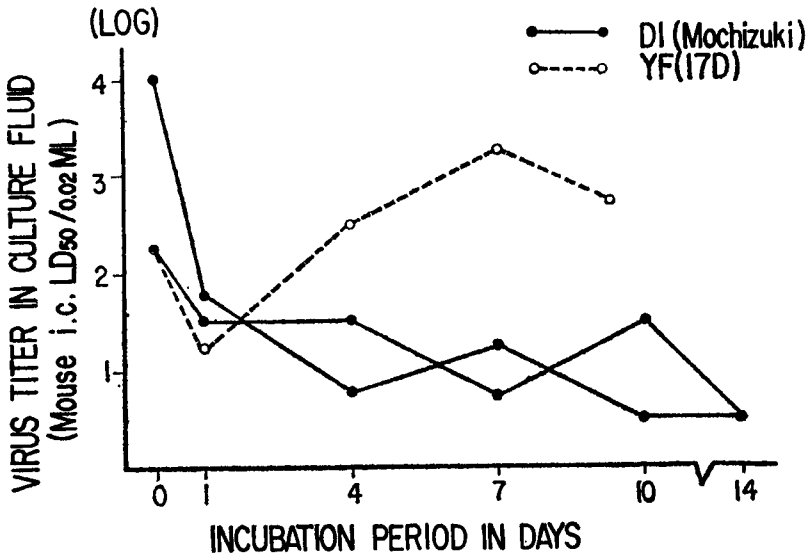

Fig. 1 Growth curves of mouse-passaged D1 and YF viruses in CESM cultures. Temperature: 37°C

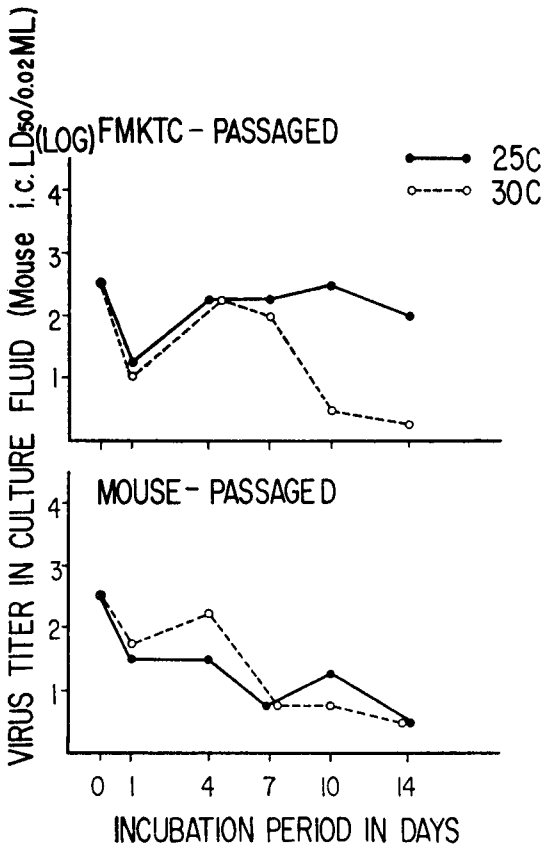

Fig. 2 Growth curves of TC and mouse-passaged D1 viruses in CESM cultures. FMKTC=Fuscatus monkey kidney tissue culture.

Chronic infection in AGMK cultures.

In dengue-infected AGMK cultures, "persistent infection" or "virus carrier state" took place. Active virus was continually detected in the fluid phase for indefinite periods of time. Similar phenomena were observed in YF-infected cultures. The whole data are summarized in Table 1, in which results up until the 400th day are included, but the infective state was maintained even longer.

The infected AGMK cells exhibited definite degeneratien. Five to 7 days after the virus inoculation, a small number of cells were dark and rounded. Such cells increased in number during the following few days and they eventually disintegrated. At about 2 weeks after the virus inoculation, one-third to one-half of the cellular sheets had been destroyed. At that time there were patchy groupings of apparently unaffected cells, from which new sheets began to develop. This process of infection was repeated so that destruction and restoration of cell sheets occurred intermittently. Plate-figures 1 to 5 inclusive show typical appearances of the chronically infected

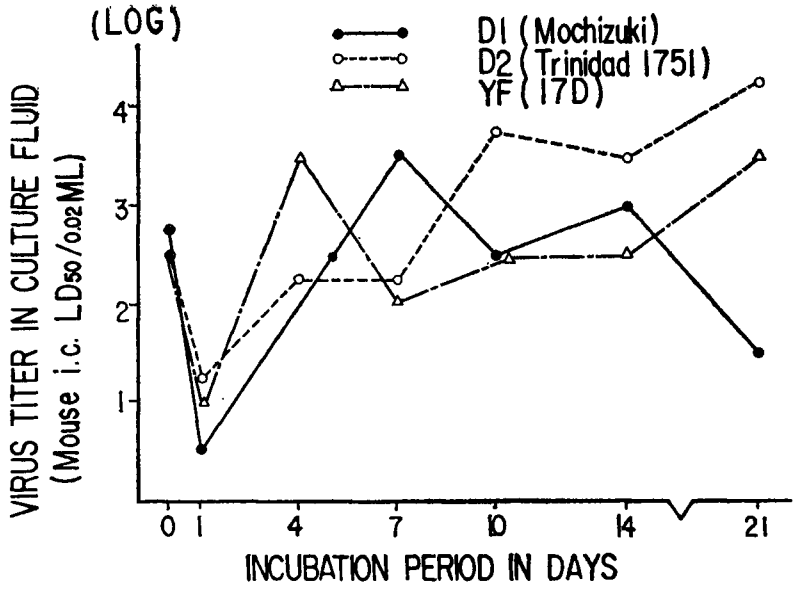

Fig. 3 Growth curves of mouse-passaged D1 and YF viruses in AGMK cultures. Temperature: 37°C

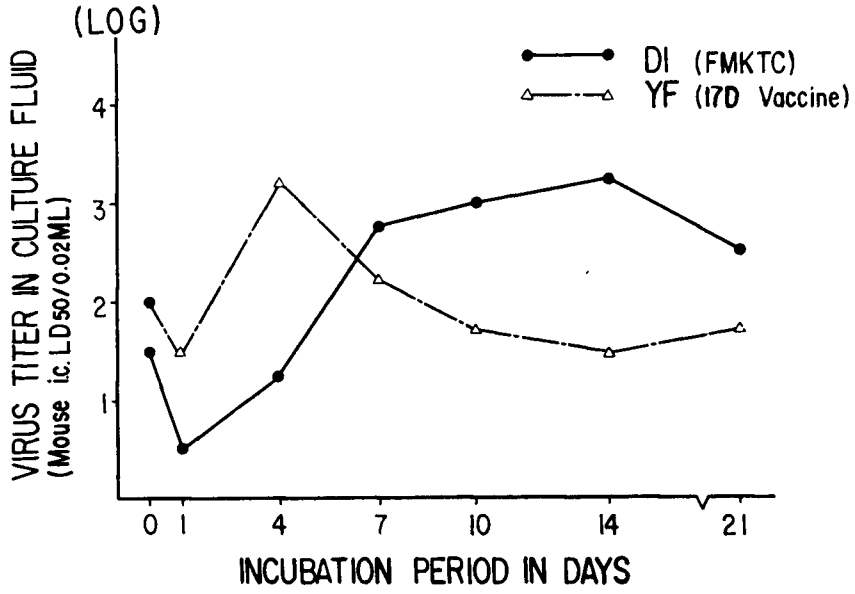

Fig. 4 Growth curves of TC-passaged D1 virus and chick embryo-passaged YF (vaccine) virus in AGMK cultures. Temperature: 37 °C.

culture cells.

DISCUSSION

In the present attempts to cultivate D1 virus in CESM cultures, multiplication of the mouse-passaged strain tested was equivocal under the conditions studied. The virus strain passed in monkey kidney cell cultures showed a little better growth in CESM cultures than did the mouse-passaged one. Even in such case, however, transmission of the virus through a series of CESM cultures was not successful. These results are remarkable, since CESM cultures can well support growth of many kinds of arboviruses. For example, YF virus which is widely believed to resemble most closely dengue virus, can multiply rather easily in CESM cultures. Our past experiences also indicate that YF (17D strain) and D1 viruses are similar in affinity to, and growth pattern in, various tissue culture systems (Hotta *et al*, 1962).[6] The difference in CESM affinity of these two viruses may be significant for distinguishing them biologically.

Table 1. Chronic infection of D1 and YF viruses in AGMK cultures.

	D1-MB	D1-FMTC	D2-MB	YF-Vaccine	YF-MB
1*	≦0.25**	0.5	1.25	1.5	1.5
4	2.5	1.25	2.25	3.25	3.5
7	3.75	2.75	2.25	2.25	2.0
10	2.5	3.0	3.75	1.75	2.5
14	3.0	3.25	3.75	1.25	2.5
21	1.5	2.5	3.5	1.75	3.5
31	1.75	2.25		1.75	2.75
41	1.75	1.75		1.75	≧2.25
58	1.5	1.5		2.5	2.5
70	≧2.5	ND	2.75	≧2.5	≧2.5
96	≧1.5	ND		≧1.5	≧1.5
130	≧1.5		2.5	≧2.5	≧1.5
160	2.25		1.5		2.25
247	≧2.5		3.5		
274	2.5		2.5	2.75	3.25
300	3.25			2.75	2.5
339	≧3.5				
358	2.75		3.5	3.25	1.5
400	3.25		3.5	3.25	2.5

MB : Mouse brain passaged.
FMTC : *Fuscatus* monkey kidney TC passaged.
ND : No active virus detected.
* Period of cultivation in days.
** Mouse intracerebral LD_{50} per 0.02 ml.

No reason of the refractoriness of CESM cultures to dengue virus is obvious. Interferon may play a role. Further studies are required to clarify the problem. Knowledge of this point is not only of basic interest but also may be useful in research for production of a "dengue vaccine". If a "CESM-adapted" strain of dengue virus were secured, it could be used as a source of "live dengue vaccine", just as achieved for yellow fever. As discussed in a separate paper (Hotta *et al*, 1966),[5] human-attenuated Mochizuki strain virus cultivated serially in monkey kidney cell cultures, when injected in the living state, can stimulate monkeys or human beings to produce anti-dengue neutralizing antibodies in considerable titers. This may open a possibility of developing a live dengue vaccine of tissue culture origin. However, utilization of monkey kidney cells for this purpose needs further considerations. As is well known, the monkey kidney tissues have a potential danger of being contaminated by agents pathogenic for humans. From this point of view CESM cells are probably safer than are the monkey kidney cells, although there is still a possibility for CESM cells to be contaminated by agents of fowl origin. On

the other hand, monkeys bred under scientific regulations may be used as a source of live vaccine.

AGMK cultures supported well the growth of both D1 and YF viruses. In this culture system, so-called "virus carrier state" or "persistent infection" was noted. Prolonged persistence of D1 virus *in vitro* was first found in experiments in which the Mochizuki strain virus survived in monkey testicular tissue cultures for at least 11 weeks (Hotta and Evans, 1956[4]; Hotta, 1959).[2] More elaborated observations of this sort have later been reported by other workers using established cell line culture systems (Wiebenga, 1961;[10] Beasley *et al*, 1961;[1] Schluze and Schlesinger, 1963[7]). The results of the present study are compatible with these previous ones. Although the cause of the phenomenon has not yet been clear, there is an impression from the data so far available that dengue virus produces a chronic infection in tissue cultures more easily than some other kinds of viruses such as polioviruses which seldom affect the same culture cells in such a manner. As to general discussions on the carrier state of animal viruses in tissue cultures, refer to a review article by Walker (1964).[9]

ACKNOWLEDGMENT

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I. Carrier culture of human skin cells infected with dengue–1 virus.
II. Cytopathogenic virus subcultured from HuS 2806 dengue–1 carrier culture.

Plate-Fig. 1

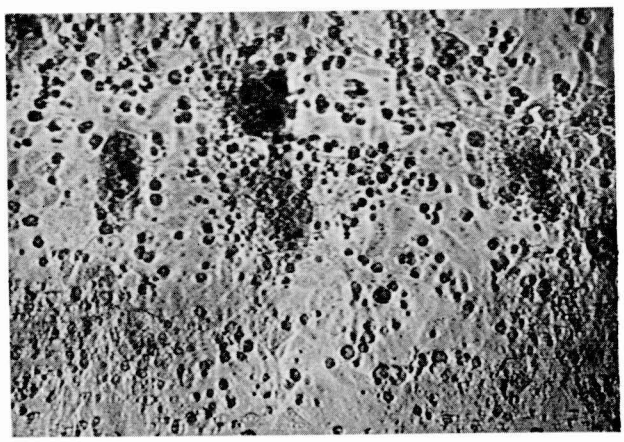

Plate-Fig. 2

Plate-Figs. 1, 2. D1-infected AGMK cultures. Twelve months after virus inoculation. Plate-Fig. 1 shows the degeneration of cells, and Plate-Fig. 2 shows the restoration of cellular sheets.

Plate-Fig. 3

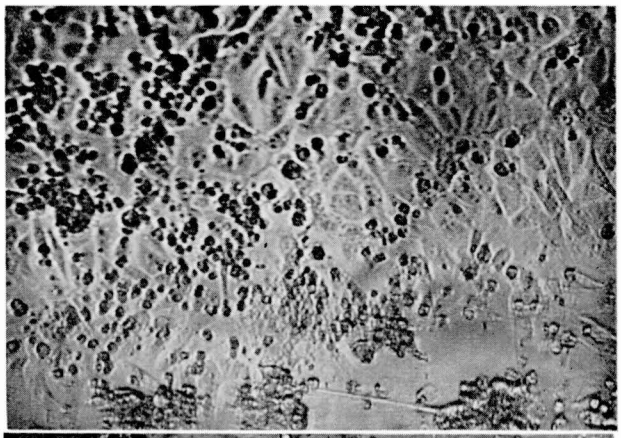

Plate-Fig. 4

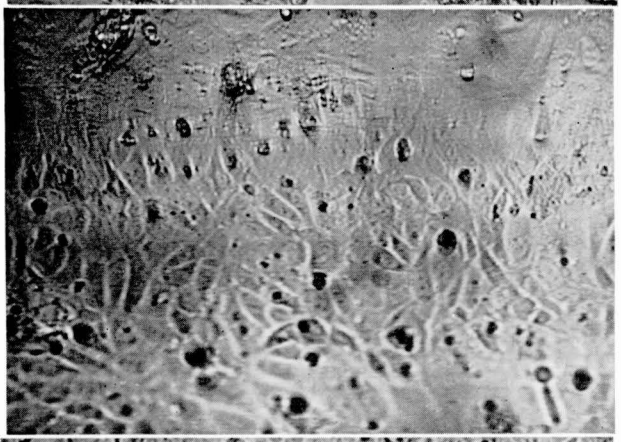

Plate-Fig. 5

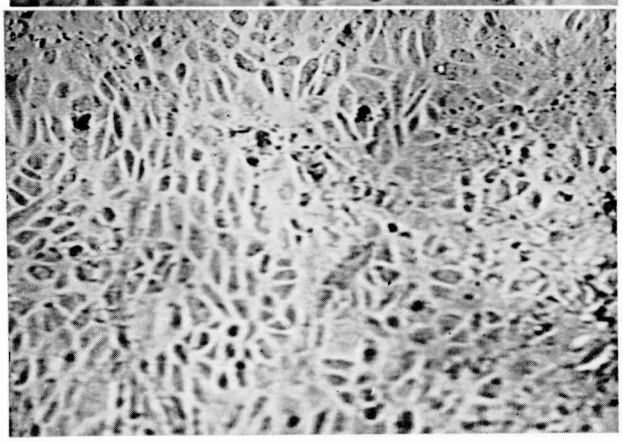

Plate-Figs. 3, 4. YF-infected AGMK cultures. Twelve months after virus inoculation. Explanations of the figures are similar to those in Plate-Figs. 1 and 2.

Plate-Fig. 5. Non-infected AGMK culture, after twelve months' incubation. No degeneration is seen.