BODY OF REPORT

SEATO Medic Study No. 10	Growth of Dengue Viruses in Tissue Culture
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Objective: To develop cell culture systems which are equally sensitive or superior to the suckling mouse for the isolation, assay and cultivation of dengue viruses.

Description: Systematic tests of available primary cell explants and stable cell lines for susceptibility to mouse adapted and wild strains of dengue viruses. Methodology includes search for CPE producing, plaque and interferon producing systems.

Progress: The requirement for a simpler, cheaper and more versatile host than the suckling mouse for the isolation, identification and growth of dengue viruses has led to intensive studies of tissue culture for the past 3 years. The first system studied, that of growing dengue viruses in tube cultures of mammalian cells using interference to virus challenge as a detection system, has been highly successful. The African grivet monkey cell line, BS-C-1, has proven to be as

Table 1

THE FORMULA OF BS-C-1 GROWTH MEDIUM

Ingredients	ml. in 100 ml. medium
Fetal bovine serum	20.0
5.0% glutamine	1.0
10.0% yeastolate	1.0
7.0% sodium bicarbonate	0.7
Medium 199	76.0
Penicillin	300 units/ml.
Streptomycin	300 mgm/ml
Mycostatin	50.0 mgm/ml

Table 2

THE FORMULA OF BS-C-1 CELL MAINTENANCE MEDIUM

Ingredients	ml. in 100 ml. medium
Heat inactivated newborn calf serum	5.0
5.0% glutamine	1.0
7.0% sodium bicarbonate	0.7
Medium 199	92.0
Penicillin	300 units/ml.
Streptomycin	300 mgm/ml.
Mycostatin	50.0 mgm/ml.

susceptible as the suckling mouse for assay of high and low mouse passage dengue viruses and acceptable as a substitute for suckling mice for recovery of dengue viruses. More recently the environmental conditions required for plaquing dengue viruses in MK 2 cells have been defined.

Basic Methods

Viruses: The mouse passage prototype viruses used in these studies were: dengue type 1, Hawaii, 126th mouse passage; dengue type 2, New Guinea, 24th mouse passage; dengue type 3, H-87, 21st mouse passage; dengue type 4, TH-241, 25th mouse passage.

Low mouse passage viruses studied were strains recovered from humans and mosquitoes between 1962-64 in Bangkok. The passage level and year of recovery are indicated below.

Tissue culture passage viruses studied were strains recovered from humans and mosquitoes in BS-C-1 cells. The specimen number, passage history and year of recovery are indicated in the appropriate table.

Tissue Culture: BS-C-1 cells. The continuous line of African grivet monkey kidney cells, BS-C-1 was used for tube method virus assay. This cell line was received in December, 1962 from Microbiological Associates, Bethesda, Maryland via Walter Reed Army Institute of Research.

BS-C-1 cell outgrowth and maintenance media used are shown in Tables 1 and 2.

For the production of cell sheets 0.5 ml. of growth medium containing 50,000 cells was seeded in screw capped test tubes (12.5 ml X 1.5 ml). After incubation at 37°C for 4-5 days, confluent cell sheets were generally formed.

LLC-MK 2 cells. A stable line of rhesus monkey kidney cells, LLC-MK 2 were employed in studies of dengue plaquing. This cell line was obtained from Dr. Robert Hull, Eli Lilly Co., Indianapolis, Indiana. Growth and maintenance media were changed from those described by Hull (5). Formulae are shown in Tables 3 and 4. For cell propagation, 7×10^5 cells were inoculated in 2 oz. prescription bottles. Confluent layers were formed in 3-4 days.

Mice. One or two-day-old mice used were from the SMRL colony. For estimation of suckling mouse titers of virus preparations, two litters of 8 suckling mice each were inoculated intracerebrally (IC) with 0.02 ml with appropriate virus dilutions. Mice were observed for sickness or death for 21 days and LD_{50} was calculated by method of Reed and Muench.

Dengue Virus Tube Assay Method. (Challenge virus resistance technique)

Dilution of viruses. Virus dilutions were made in medium 199 with 5.0% heat-

Table 3

THE FORMULA OF LLC-MK2 CELL GROWTH MEDIUM

Ingredients	ml. in 100 ml. medium
Calf serum	20.0
10.0% yeastolate	1.0
5.0% glutamine	1.0
Tryptose phosphate broth	15.0
7.0% sodium bicarbonate	0.7
Medium 199	61.0
Penicillin	300 units/ml.
Streptomycin	300 mgm/ml.
Mycostatin	50.0 mgm/ml.

Table 4

THE FORMULA OF THE FIRST AGAR OVERLAY FOR LLC-MK2 CELLS.

Ingredients (2X BME)	ml. in 100 ml. medium
Heat inactivated calf serum	20.0
100% vitamin BME	2.0
100% amino acids BME	2.0
5.0% glutamine	1.2
4.0% sodium bicarbonate	6.0
Hank's BSS (no phenol red)	62.0
Penicillin	600 units/ml.
Streptomycin	600 mgm/ml.

Add equal parts of above medium (heated to 37° C) to 2% Noble agar (42°C).

Table 5

SAMPLE PROTOCOL OF VIRUS TITRATION BY THE CVR METHOD IN BS-C-1 CELL CULTURE

Dengue 3 virus dilutions (neg $\log_{10}$)	No. tube	Day of Observation 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
5.0	1	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
	2	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
	*Control	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
6.0	1	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
	2	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
	Control	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
7.0	1	x	x	x	x	x	x	x	x	x	x	1	4			
	2	x	x	x	x	x	x	x	x	x	x	x	2	4		
	Control	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
8.0	1	x	x	x	x	x	x	x	x	x	x	2	4			
	2	x	x	x	x	x	x	x	x	x	x	2	4			
	Control	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x

Medium change

Challenge with 100 TCD_{50} ($10^{-3.5}$) of Polio 1, titer $10^{-5.5}$

CVR = 6.5 (neg $\log_{10}$ InD_{50})

Challenge virus check titration

Polio I virus dilution Neg. $\log_{10}$		Day of observation 1	2	3	4	5	6	7	8
3.0	1	x	x	x	2	4			
	2	x	x	x	2	4			
	3	x	x	x	2	4			
	4	x	x	x	2	4			
4.0	1	x	x	x	1	4			
	2	x	x	x	x	2	4		
	3	x	x	x	x	2	4		
	4	x	x	x	x	2	4		
5.0	1	x	x	x	x	x	2	4	
	2	x	x	x	x	x	1	4	
	3	x	x	x	x	x	2	4	
	4	x	x	x	x	x	2	4	
6.0	1	x	x	x	x	x	x	x	x
	2	x	x	x	x	x	x	x	x
	3	x	x	x	x	x	x	x	x
	4	x	x	x	x	x	x	x	x

Polio I titer = 5.5 (neg. $\log_{10}$)

* The third tube of each dilution not inoculated with polio I virus.

inactivated new-born calf serum, adjusted to pH 7.2-7.4 with sodium bicarbonate (M 199-CS). Two ampoules containing 20% virus-mouse brain suspension or tissue culture fluid were pooled for each dengue virus titration.

Inoculation of tubes. Growth medium was poured off the cell sheet just prior to inoculation. Then, 0.1 ml of inoculum was added into each of 3 tubes. Inoculated tubes were incubated in a slant position for 2 hours at 34° C after which 1.0 ml of maintenance medium was added. Tubes were incubated at 34° C. Media were changed on the 5th day and pH was adjusted at frequent intervals to pH 7.2-7.4 with 4.0% sodium bicarbonate. Cells were examined daily for cytopathic effect (CPE).

Challenge of infected tubes. To detect the presence of dengue virus in cell cultures, 2 tubes at each dilution were challenged on the eighth post-inoculation day with approximately 100 TCD_{50} of a second challenge virus. Challenge virus inocula (0.1 ml) were added directly to maintenance medium. Challenged tubes were unchallenged and observed for 16 days for direct dengue induced CPE.

Challenge virus test. In preliminary studies comparing the utilization of polio and chikungunya viruses as the challenge agents in assay of high mouse passage strains of dengue 1-4 only one difference was noted. Chikungunya virus produced 4+ CPE more rapidly than polio type 1 did and thereby shortened the time necessary for the test. The test was read 24 hours after tubes inoculated with only challenge virus showed 4+ CPE. Tubes inoculated with test material and

challenge virus which showed only 0 to 1+ CPE at that time were considered to be CVR positive. That is to say that they showed interference effect. The number of CVR+ tubes were used to calculate the ID_{50}. Table 5 shows a typical completed protocol of a dengue virus titration using the CVR method.

Dengue Plaques

Virus dilution: For plaque studies, dengue viruses were diluted with M 199-CS, adjusted to pH 8.0 with 7.0% sodium bicarbonate.

Inoculation of bottles. Cell sheets were washed once with Hank's BSS 8.0, without phenol red. Then, 0.5 ml of each virus dilution was inoculated onto cell sheets. Bottles were rocked manually for 5-10 minutes. Following that, inoculated bottles were incubated at 37°C for 2 hours with manual rocking at 15 minute intervals. The cell sheet was washed again with Hank's BSS, then overlaid with 4.0 ml of a mixture of Noble agar and Eagle's medium. After solidification of agar, bottles were incubated at 37° C in an inverted position.

First overlay. The first overlay consists of equal volumes of 2.0% Noble agar containing 0.01% DEAE dextran and 2X Basal Medium Eagle (BME) (Table 4). Noble agar was prewarmed at 42° C and mixed with medium at 37° C.

Second agar overlay and neutral red staining of cell sheets. On the 7th day after original inoculation, a second agar overlay containing neutral red in a final concentration of 1:10,000 and BME (IX) without calf serum or sodium bicarbonate was added to each bottle.

Reading plaques. Plaques are counted on the 8th day. Some instances, additional incubation at 37° C for 1-3 days increased plaque size and plaque sharpness.

Results:

Challenge virus resistance test. Results of CVR titrations of high and low mouse passage viruses show that BS-C-1 cells gave titration results with all dengue strains which were equal to or better than results obtained by intracerebral inoculation of suckling mice (Table 6).

Plaque studies pH during period of virus absorption. When pH of virus diluent was maintained between 8.0- to 9.0, plaque counts were higher and more distinct than when lower pH was maintained during this period.

pH during virus growth phase. When Eagle's medium was adjusted with sodium bicarbonate to various pH's ranging from 7.0 to 8.5, media with pH between 8.0-8.2 resulted in the highest plaque counts and the most distinct plaques. A pH higher than 8.5 resulted in a change of the color of neutral red from red to yellow, not allowing visual discrimination of plaques.

Table 6

RESULTS OF CVR TITRATIONS OF SELECTED HIGH AND LOW MOUSE PASSAGE DENGUE VIRUSES IN BS-C-1 CELLS.

Viruses	Suckling mouse passage	Dengue type	Neg $Log_{10}LD_{50}$*	Neg $Log_{10}InD_{50}$**
Deng. 1 (Hawaii)	70		7.3	6.5
Deng. 2 (New Guinea C)	26		8.1	9.0
Deng. 3 (H-87)	21		7.3	7.5
Deng. 4 (H-241)	25		6.8	7.5
TH-36	14		8.2	8.5
TH-Sman	14		7.0	7.0
BKM 48-62	3	2	7.2	6.5
BKM 59-62	3	2	7.2	6.5
BKM 106-62	3	2	6.9	6.5
1566-62	3	3	5.2	4.5
2189-62	3	1	5.9	5.5
5355-62	3	1	5.2	6.0

* LD_{50} = 50 per cent mouse lethal dose by intracerebral titrations.

** InD_{50} = 50 per cent tissue culture infectious dose based on tubes which resist 100 TCD_{50} of challenge virus.

Effect of concentration of calf serum in first overlay. Medium with 2.5% calf serum resulted in only minute and indistinct plaques; plaques produced under 5.0% calf serum were slightly larger, while large and distinct plaques were formed in media containing 10.0% or 20.0% final concentrations of calf serum.

Day of addition of neutral red. It has been shown previously in plaquing dengue 2 virus in KB cells that the addition of neutral red earlier than the fifth day suppressed the plaque count. Similiar results were obtained in our study. Addition of neutral red (1:10,000 final dilution) overlay earlier than the fifth day resulted in minute plaque formation or complete suppression of plaque formation.

Effect of DEAE dextran. Addition of DEAE dextran to a final concentration of 0.005 per cent resulted in production of more distinct plaques, but did not affect the total number of plaques. Increase in concentration above 0.005 per cent did not improve plaque results.

Miscellaneous. Plaquing of dengue 4 virus in Eagle's medium (BME) of normal strength resulted in optimal plaque production. Four fold increase in vitamin or glutamine, or 4-fold decrease in amino acid concentrations inhibited plaque formation. Four-fold variation of Ca^{++} and Mg^{++} in the 1st overlay did not effect plaque count, size or sharpness.

Plaquing of high mouse passage dengue virus. Dengue 2, New Guinea C strain produces 2 mm distinct plaques with sharp margins; dengue 3 (H-87) virus produces large plaques 2-3 mm in diameter, (further incubation increases plaque

size producing serrated edges and a fluffy appearance); dengue 4 (TH-241) plaques average 2 mm in diameter and have rather regular edges (no change in size is noted on additional incubation). Plaques with 126th mouse passage, dengue 1 (Hawaii) are very tiny and indistinct; plaques with 70th mouse passage Hawaii strain are somewhat bigger while a 3rd mouse passage dengue 1 (13505) produced distinct plaques of 2.0 mm size.

Plaquing of low mouse passages viruses. Many of the low mouse passage viruses thus far examined are mixtures of large and small plaques.

Plaquing of tissue culture isolates. Tissue culture isolates when plaqued frequently consisted of plaques of homogeneous size. Comparative studies of plaque titration and mouse titration of high mouse passage, low mouse passage, and tissue culture passage dengue viruses (BS-C-1), CVR method, are shown in Table 7.

Summary and Conclusions: The methodology for titering dengue viruses in tube cell cultures and for plaquing dengue viruses has been presented briefly. Using an interference method, BS-C-1 cells are as sensitive as the suckling mouse for the titration and primary recovery of dengue viruses. A sensitive plaque assay has been developed using a continuous rhesus kidney cell line, LLC MK 2. Plaques may provide not only visual virus genetic markers, but, allow differentiation of other viral characters, such as temperature or pH variants. Further, it may become possible to separate mixtures of virus types or to clone viruses selected progeny for analysis of biologic or antigenic variants. Both BS-C-1 and MK 2 cells have shown very desirable characteristics for long term use in the laboratory. Both propagate rapidly, require relatively low cell inocula to obtain good growth, have been stable in their virus susceptibilities. Cells are routinely frozen at -70° C and cell lines re-established every 6 months. Although large amounts of serum are apparently needed in maintenance media for good dengue virus growth, it is hoped that less expensive media can be found, thus adding economy to the attributes of versatility and sensitivity already inherent in dengue virus tissue culture methods.

Publications:

Halstead, S. B., Sukhavachana, P., Nisalak, A., 1964. Assay of mouse adapted dengue viruses in mammalian cell cultures by an interference method. Proc. Soc. Exp. Biol. Med. 115: 1062-1068.

Halstead, S. B., Sukhavachana, P., Nisalak, A., 1964. *In vitro* recovery of dengue viruses in naturally infected human beings and arthropods. Nature, 202: 931-932.

Table 7

COMPARATIVE TITERS OF MOUSE PASSAGE DENGUE VIRUSES AND TISSUE CULTURE ISOLATES (BS-C-1 CELLS) OBTAINED BY PLAQUE, CVR AND MOUSE TITRATION.

Specimen No.	Origin of specimen	Host and passage level	Tentative identification	Virus titers in 0.1 gm. of material (neg $\log_{10}$)		
				PFU	InD_{50} *	LD_{50} **
Dengue 1 (Hawaii)		SMP 70		6.7	6.5	7.3
Dengue 2 (New Guinea C)		SMP 26		6.9	9.0	8.1
Dengue 3 (H-87)		SMP 21		6.3	7.5	7.3
Dengue 4 (TH-241)		SMP 25		6.3	7.5	6.3
BKM 85-62 *	Mosquito	SMP 6	4	7.9	8.5	7.4
BKM 68-62	Mosquito	SMP 4	2	5.8	7.5	6.3
BKM 475-62	Mosquito	SMP 5	3	5.7	5.5	5.8
3149-62	Human	SMP 4	1	5.6	6.5	6.0
6141-62	Human	TC ***	2	3.5	3.8	3.0
13283-(64)	Human	TC	1	3.3	3.8	3.8
BKM 331-62	Mosquito	TC	3	6.1	6.5	6.4
13508-(64)	Human	TC	Uniden.	4.0	3.8	3.8

+ = Year of recovery
* = 50 per cent tissue culture infectious dose based on tubes which resist 100 TCD_{50} of challenge virus.
** = 50 per cent mouse lethal dose by intracerebral titration.
*** = Tissue culture isolated in BS-C-1 cells.