

ANNUAL PROGRESS REPORT

SEATO Medic Study No. 10 - Growth of Dengue Viruses in Tissue Culture
Project No. 3A 025601 A 811 Military Medical Research Program
S.E. Asia
Task 01: Military Medical Research Program
S.E. Asia
Subtask 01: Military Medical Research Program
SEASIA (Thailand)
Reporting Installation: US Army-SEATO Medical Research Laboratory
APO 146, San Francisco, California
Division of Medical Research Laboratories
Department of Virology
Period Covered by Report: 1 April 1963 to 31 March 1964
Principal Investigator: Major Scott B. Halstead, MC
Associate Investigators: Dr. Pairat Sukhavachana
Dr. Ananda Nisalak
Assistant Investigator: SSG Merlyn J. Funkenbusch
Reports Control Symbol: MEDDH-288
Security Classification: UNCLASSIFIED

ABSTRACT

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The objective of this study is to develop a single cell culture system which is equally sensitive or superior to the suckling mouse for the isolation, assay and cultivation of dengue viruses. During the report period considerable progress has been made in the assay and recovery of dengue viruses in cell cultures. Using standard growth and maintenance media, 4 cell lines and 2 primary cell cultures were tested as assay system for prototype dengue viruses of suckling mouse origin. BS-C-1 cells, a stable line of grivet monkey kidney cells was found to be equally sensitive

as the suckling mouse for assay of dengue 1-4, TH 36 and TH Sman using as end point the ability of dengue infected cell sheet to resist CPE due to challenge virus. MK2, a stable line of Rhesus kidney cells, were slightly less sensitive, PS, a pig kidney line, hamster kidney cells and primary monkey kidney cells were only moderately sensitive, and a strain of Hela was insensitive for dengue assay using the interference technique. Low mouse passage dengue viruses are readily titered in BS-C-1 cells; neutralization tests have been successfully accomplished in this system and dengue virus typing using BS-C-1 cells is now underway. Comparative studies of the 1 day old suckling mouse and BS-C-1 cells for primary recovery of dengue viruses have been made. BS-C-1 cells were only slightly less efficient for recovery of dengue viruses of human origin than were suckling mice. Of mosquito suspensions tested some viruses were recovered in suckling mice only while others were isolated only in tissue culture. Study of this phenomenon is in progress. Interference in BS-C-1 infected cells is correlated with the development of a substance with properties similar to the interferon of Isaacs. Studies of nutritional requirements for maximal growth of dengue in cell cultures are in progress. Plaque work with dengue virus is under study.

BODY OF REPORT

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Objectives: To develop a single cell culture system which is 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: Studies of Dengue Viruses in Tissue Culture

Until recently the suckling mouse has been the only satisfactory host for the isolation, growth and identification of dengue viruses. Work with this host has been inefficient, time consuming and expensive. Primary isolation of virus from human and arthropod sources usually requires several passages in mice before dengue virus is sufficiently neurovirulent to kill mice reproducibly and in some instances prolonged passage is required before usable titers are achieved. These difficulties have encouraged workers to examine cultured cells as alternate hosts. Until the present study no cell system had been found to be as sensitive as the suckling mouse to all dengue virus prototypes. Recent demonstration of interference phenomena in virus infected cells have led to the development in this laboratory of the challenge virus resistance test (CVR). Application of this technique to BS-C-1 cells, a stable African grivet monkey kidney line, has provided a system of assay for all prototype dengue viruses of equal or superior sensitivity to the suckling mouse.

Tissue Culture and CVR Test Methods

Tissue cultures are grown in stationary screw cap test tubes (12.5x1.5cm). When ready for use, the uncovered cell sheet of each of 4 tissue culture tubes is inoculated with 0.1 ml amounts of specimen preparation. Tubes are incubated at 34 C for 2 hr., then the inoculum is removed and 1 ml of maintenance medium is added (for mosquito suspensions cell sheet was washed with maintenance medium before adding final maintenance medium). Maintenance medium is changed on the fifth day and cells observed daily for cytopathic effect (CPE). Eight days after the original inoculation, approximately 100 TCD₅₀ of challenge virus is added to each of 2 of inoculated tubes and to not less than 10 uninfected control tubes. Development of only 0-1+ CPE 24 hours after challenge virus control tubes showed 4+ CPE is considered evidence of resistance to challenge virus (CVR+). For passage the unchallenged tubes are routinely quick frozen and thawed for 3 cycles. They are routinely passed to third passage after 10-12 days incubation at each passage level. Unchallenge companion tubes of CVR positive tubes were passaged to 6 tissue culture tubes to produced seed virus. Two of these tubes are inoculated with challenge virus to verify challenge virus resistance. Dengue virus is harvested from positive tubes after 10-12 days incubation.

The various cell lines tested for growth of dengue virus were:

<u>Cell line</u>	<u>Type</u>	<u>Origin</u>	<u>From</u>	<u>Challenge Virus</u>
GMK (BS-C-1)	Stable	African green monkey kidney	NIH, Bethesda	Polio 1
MK2 (LLC-MK2)	Stable	Rhesus monkey kidney	Dr. R.N.Hull Eli Lilly Rsch Laboratory	Polio 1

<u>Cell line</u>	<u>Type</u>	<u>Origin</u>	<u>From</u>	<u>Challenge Virus</u>
PS	Stable	Porcine kidney	NIH, Japan	Coxsackie B 1
HKC	Primary	10 day old hamster		Chikungunya
MK	Primary	<u>Macaca irus</u> kidney		Polio 1

Mechanism of Interference

Challenge virus resistance in dengue infected cells is thought to be due to production of interferon-like substances. Dengue-BS-C-1 interferon has a titer of 1:64-1:128 and is cell species specific, but when incubated with dengue virus and inoculated on uninfected homologous cells will not prevent infection. Selected properties of BS-C-1 "interferon" are shown in Table 45.

Assay of High Mouse Passage Dengue Viruses

Using high mouse passage suckling mouse brain virus, a number of cell lines were tested for sensitivity to prototype dengue viruses. Only BS-C-1 cells gave results equal to or better than suckling mice (Table 46). Tissue culture tubes inoculated with very small dengue virus inocula at limit dilutions were CVR positive and dengue virus could be recovered in mice from these tubes.

Assay of Low Mouse Passage Dengue

Low mouse passage dengue recovered from man and mosquitoes are even more readily titered in tissue culture than suckling mice. Results of testing a large number of dengue viruses at 2nd to 4th mouse passage are shown in table 47. The superiority of the 2 monkey kidney cell lines is evident. Representative titers achieved with low mouse passage material in several cell lines is demonstrated in Table 48.

Primary Isolation of Dengue Viruses in Tissue Culture

Success with high and low mouse passage dengues led to an examination of cell culture systems for the isolation of dengue virus. Specimens originally positive in mice were taken from the Revco and inoculated in cell cultures.

Comparative results of dengue virus isolations from human sera and mosquito suspensions in tissue culture using 4 laboratory cell lines are presented in Table 49. The dengue virus sensitivity of BS-C-1 and MK2 do not greatly differ, but the monkey kidney lines are distinctly superior to PS and HKC.

Table 50 shows the comparative results of isolation in suckling mice and in tissue culture. The efficiency of reisolation from human sera was 78.8% in suckling mice versus 69.2% for BS-C-1 cells. Isolation from positive

mosquito suspension, 48.7% for suckling mice and 41.0% for BS-C-1 cells. Isolation from positive mosquito suspension, 48.7% for suckling mice and 41.0% for BS-C-1 cells. As shown in the Table, virus was not recovered in every specimen. Possible reasons for this are:

1. Specimens were stored for long periods of time and virus may have become inactivated.
2. The results of first isolation may have been incorrect.
3. Specimen was in short supply and had to be diluted for reisolation attempt. This dilution may have exceeded the original concentration of virus in the specimen.

Further analysis of results of isolation of human sera and mosquito suspensions in suckling mice and in BS-C-1 cells are presented in Table 51. For human sera 41 of total of 43 viruses recovered were isolated in suckling mice; 36 viruses were isolated in BS-C-1 cells, 34 of these isolated in both suckling mice and BS-C-1 cells, 7 isolated in suckling mice only and 3 isolated in BS-C-1 cells only. For mosquito suspensions, a total of 26 viruses were isolated in both systems, 19 of them in suckling mice and in BS-C-1 cells, 16 isolated in BS-C-1 cells. Nine viruses were isolated in both suckling mice and BS-C-1 cells, 10 isolated in suckling mice only and 7 isolated in BS-C-1 cells only. This suggests that each system may be slightly more efficient for certain virus strains or virus types. Investigation into the reason for these variations is underway.

Table 52 shows the passage level of primary isolation of dengue viruses in suckling mice and in BS-C-1 cells. Most viruses in suckling mice were isolated in second or third passage, while most viruses isolated in BS-C-1 cells were at passage 1 or 2.

During the experiment, some serum specimens were found to be CVR positive on first passage but not at second or third. This may be explained by neutralization of challenge polio virus by some human serum specimens with high titered polio antibody. To avoid the possibility of false positive reaction the schedule of isolation in BS-C-1 cells was changed to that shown below:

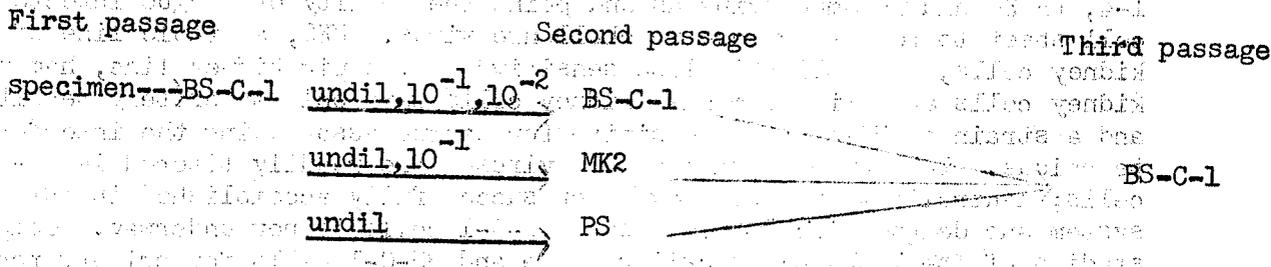
First passage	Second passage	Third passage
4 tubes no challenge	6 tubes	6 tubes (seed)
all 4 tubes	(2 tubes, challenged)	(2 tubes, challenged)

Further reason for adopting routine blind passage of all first passage tubes is that only one of four tubes may be infected with low titered inocula. If this tube were selected for challenge and negative tubes passed, no virus would be recovered in second passage.

A major problem remained to be studied. If interferon was routinely produced by dengue infected BS-C-1 cells, what did this interferon do to dengue virus when passed to uninfected cells?

In order to test the effect of interferon on growth of dengue virus, BS-C-1 tubes which contained first passage dengue virus were passed to the second passage in various cell lines at various dilutions as shown in the scheme:

Scheme of Dengue Interference Experiment



The purpose of this scheme is to dilute beyond interferon effect or to avoid interferon effect by changing to new cell species. BS-C-1 infected fluid was inoculated at undiluted, 10^{-1} and 10^{-2} to BS-C-1 cells, at undiluted and 10^{-1} to MK2 cell. In PS, a pig kidney stable cell line only undiluted fluid was inoculated. All second passages were passed back to BS-C-1 at undiluted concentration for third passage. (Table 53)

The results show that dengue virus was recovered readily from undiluted TC fluid and that dilution lowered the efficiency of recovery of dengue virus rather than raised it. It was concluded that BS-C-1 interferon was ineffective in preventing infection of BS-C-1 cells with dengue virus when dengue virus interferon mixtures were inoculated simultaneously.

Dengue viruses isolated in tissue culture have been titered in BS-C-1 cells by the CVR method. Unfortunately, tissue culture titers of new isolates are rather low in comparison with titers of suckling mouse isolates. Titers of human plasma isolates were between $10^{-2.5}$ to $10^{-4.5}$, titers of mosquito isolates were between $10^{-1.5}$ to $10^{-3.0}$. To obtain high titers, two methods are being tried. Specimens are repeatedly passed in BS-C-1 cells; other specimens are passed into suckling mice and then back to tissue culture.

Identification of dengue virus isolated in tissue culture has been done extensively with suckling mouse isolates. Confirmatory tests are repeated with tissue culture isolates. Selected results of tissue culture neutralization tests are shown in Table 54.

The data presented show that naturally occurring dengue virus of several serotypes may be recovered in BS-C-1 cells using an interference technique for recognition of cellular infection.

Summary and Conclusions: During the report period considerable progress has been made in the assay and recovery of dengue viruses in cell cultures. Using standard growth and maintenance media, 4 cell lines and 2 primary cell cultures were tested as assay system for prototype dengue viruses of suckling mouse origin. BS-C-1 cells, a stable line of grivet monkey kidney cells, was found to be equally sensitive as the suckling mouse for assay of dengue 1-4, TH 36 and TH Sman using as end point the ability of dengue infected cell sheet to resist CPE due to challenge virus. MK2, a stable line of Rhesus kidney cells, were slightly less sensitive; PS, a pig kidney line, hamster kidney cells and primary monkey kidney cells were only moderately sensitive, and a strain of Hela was insensitive for dengue assay using the interference technique. Low mouse passage dengue viruses are readily titered in BS-C-1 cells; neutralization tests have been successfully accomplished in this system and dengue virus typing using BS-C-1 cells is now underway. Comparative studies of the 1 day old suckling mouse and BS-C-1 cells for primary recovery of dengue viruses have been made. BS-C-1 cells were only slightly less efficient for recovery of dengue viruses of human origin than were suckling mice. Of 26 mosquito suspensions tested roughly 1/3 were recovered in suckling mice while another 1/3 were isolated only in tissue culture. Study of this phenomenon is in progress. Interference in BS-C-1 infected cells is correlated with the development of a substance with properties similar to the interferon of Isaacs. Studies of nutritional requirements for maximal growth of dengue in cell cultures are in progress. Plaque work with dengue virus is underway.

Table 45. Comparison of selected physical properties of Isaacs' interferon with BS-C-1 interfering substance found in tissue culture fluid removed from dengue virus infected cells.

	BS-C-1 interfering Substance	"Interferon"
Not sedimented at 100,000 g.	+	+
Resistant to pH 2.0	+	+
Destroyed by trypsin	+	+
Heat stable (56°C.)	+	+

Table 46. Maximal titers of high mouse passage prototype dengue viruses in various cell lines using an interference technique.

Dengue virus type	Mouse passage	Suckling mouse	Tissue culture virus titer					
			MKC	HKC	HeLa	MK2	PS	BS-C-1
1	73	6.7	0	0	0	6.5	3.5	7.5
2	68	7.3	3.5	5.5	0	6.5*	7.5*	6.5
3	35	6.5	3.5	5.2	0	4.5	3.5	6.5
4	35	7.3	3.5	0	0	6.5	2.5	7.5
5 (TH-36)	15	8.0	ND	6.5	0	6.5*	7.5*	8.5
6 (TH-Sman)	15	5.9	ND	4.5	0	5.5	4.0	7.0

* CPE

Table 47. Summary of comparative assay of low mouse passage dengue viruses in several cell systems by the CVR technique.

Cell culture	No. positive No. tested	Percentage positive
BS-C-1	98/103	95
MK2	14/15	94
PS	52/85	61
HK	58/102	51

Table 48. Comparative assay of selected low mouse passage dengue viruses in several cell systems by the CVR method.

Specimen	Mouse passage	BS-C-1	MK2	PS	HK
BKM-59	2	6.5	7.5*	7.5*	6.5
BKM-331	3	5.5	4.5*	2.5	3.5
BKM-418	3	6.5	7.5*	6.5*	4.5
2358	4	6.5	6.5*	6.5*	5.5
5355	3	6.0	4.5	4.5	0
BKM-85	2	6.5	4.5*	1.5	0
BKM-60	3	6.5	5.5*	0	4.2
3149	3	5.0	ND	0	3.5
BKM-475	2	5.0	3.5	0	0
5031	3	5.0	6.0	0	0

* CPE

Table 49. Dengue virus isolation from human sera and mosquito suspensions by an interference method in various cell lines.

Cell line	Human serum			Mosquito suspension		
	No. tested	No. isol.	%	No. tested	No. isol.	%
HKC	24	2	8.3	36	0	0
PS	24	4	16.7	ND	ND	ND
MK2	24	13	54.2	36	12	33.3
BS-C-1	24	16	66.7	36	16	44.4

Table 50. Comparison of suckling mice and BS-C-1 cells for dengue virus isolation from human sera and mosquito suspensions.

Method Used	Human serum			Mosquito suspension		
	No. tested	No. isol.	%	No. tested	No. isol.	%
SM	52	41	78.8	39	19	48.7
BS-C-1	52	36	69.2	39	16	41.1

Table 51. Comparative efficiency of isolation of dengue viruses from original materials in suckling mice and BS-C-1 cells.

	Total isolated in SM or BS-C-1	No. isolated in both SM and BS-C-1	No isolated in SM only	No. isolated in BS-C-1 only
Human	43	34	7	2
Mosquito	26	9	10	7

Table 52. Passage level of primary isolation of dengue viruses in suckling mice and BS-C-1 cells.

Type of specimen	Suckling mouse passage				BS-C-1 cell passage					
	1	2	3	Total	1	2	3	4	5	Total
Human	2	20	19	41	23	10	2	-	1	36
Mosquito	-	1	18	19	7	8	-	1	-	16

Table 53. Effect of change of cell line and dilution of dengue virus containing tissue culture fluid on recovery of dengue in 2nd and 3rd tissue culture passage.

Specimen No. first passage	Interference to challenge virus in indicated cell line virus dilution and virus passage																	
	Second passage						Third passage in BS-C-1 from											
	BS-C-1			MK2		PS												
Undil.	10 ⁻¹	10 ⁻²	Undil. 10 ⁻¹		Undil.	a	b	c	d	e	f	a	b	c	d	e	f	
4745-62	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4128-62	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+
4162-62	+	+	±	±	±	±	±	±	-	-	-	+	±	±	±	±	±	-
1850-62	+	+	±	±	±	±	±	±	-	-	-	+	+	+	+	+	+	+

+ Virus isolated.

± Equivocal evidence of virus infection.

- No virus isolated.

Table 54. Identification of selected dengue viruses isolated in suckling mice and in tissue culture.

Specimen No.	Origin	Host passage	Log neutralization index of unknown virus vs. prototype dengue antisera						Remarks
			Dengue 1	Dengue 2	Dengue 3	Dengue 4	Dengue 5 (TH-36)	Dengue 6 (TH-Sman)	
2358	Human plasma	SM4 TC3	0	3.0 3.0	0	0	2.5 2.0	0	Dengue 2 or TH-36
BKM-418	<u>Aedes aegypti</u>	SM5 TC3	0	3.0 2.7	0	0	2.5 2.7	0	Dengue 2 or TH-36
LNI vs. homologous dengue virus			3.0	3.0	2.0	4.0	2.5	3.4	

- Publications:
1. Halstead, S.B., Sukhavachana, P. and Nisalak, A.
Assay of Mouse Adapted Dengue Viruses in Mammalian
Cell Culture by an Interference Method.
Proc. Soc. Exp. Biol. Med. In Press.
 2. Halstead, S.B., Sukhavachana, P. and Nisalak, A.
In Vitro recovery of dengue viruses from naturally
infected humans and arthropods. NATURE. In Press.