Relationship of Temperature to the Replication of Dengue Viruses in LLC-Mk_2 Cell Cultures

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OBJECTIVES : To determine the plating efficiency, plaque morphology and kinetics of replication of dengue viruses at different temperatures.

BACKGROUND : Each of the currently recognized serotypes of dengue viruses has been associated with disease of man that differs widely in severity (1, 2). One hypothesis that has been advanced to explain the variation in severity of disease is that dengue viruses differ in regard to virulence properties (3). A corollary to this hypothesis stated that the determinant of severe dengue syndrome was related to the surface antigens of dengue viruses. Although the antigenic and biological properties of these viruses differ, such markers have not proven to be associated with a particular human disease syndrome (4, 5).

Investigations have shown that the replication of dengue viruses as well as strains of each serotype appeared to be influenced by temperature (4). In addition, more recent studies have revealed that dengue viruses, especially dengue-2 were comprised of subpopulations that differed considerably in regard to *in vitro* replication at different temperatures (6). Virulence for mice was found to vary according to the temperature that was permissive for the replication of subpopulations of this virus. This study was initiated to determine if temperature requirements for *in vitro* replication of dengue viruses could be used as a marker to differentiate these viruses in regard to their pathogenicity for man.

METHODS : Wild dengue viruses were obtained in conjunction with other studies from clinically diagnosed dengue fever and dengue hemorrhagic fever patients admitted to the Children's Hospital, Bangkok. Two dengue-3 viruses were isolated from patients admitted to the Khonkaen Hospital, Khonkaen. If possible, strains of each dengue virus serotype were obtained, including strains from patients who had grade I illness and strains from patients who had grade IV and/or from fatal cases. The clinical history and the identity of each dengue virus was obtained from clinical records maintained in the Department of Virology, AFRIMS. The plating efficiency of each virus was determined by assay of original and/or $\log_{10}$ dilutions of leukocyte specimens by the direct plaque technique at 32, 35 and 39°C in LLC-Mk_2 cell cultures. The number of plaque forming units (PFU) and the diameter of plaques were recorded for viruses at each temperature.

Virus replication curves were established for selected low passage suckling mouse brain propagated dengue viruses at 32°C, 35°C and 37°C. Tube cultures of LLC-Mk_2 cells were inoculated with a concentration of virus to yield a multiplicity of infection of 0.1. At 12 hour intervals, glass beads were added to two or more cultures for each temperature. Cell cultures were then disrupted by vigorous agitation employing a vortex mixer. After adding 0.5 ml of fetal calf serum (heat treated at 56°C x 30 min) to each suspension, the content of each tube were pooled and 0.5 ml aliquots were stored at -70°C. Suspensions were assayed for virus in LLC-Mk_2 cells by direct plaque assay. Plaque forming units were recorded on day 6 post inoculation.

RESULTS : Eleven original human plasma and/or leukocyte suspensions from which dengue viruses had been isolated during 1977 were selected for plating efficiency studies. Included were strains of dengue 2, 3 and 4 viruses that were associated with different grades of human illness. Dengue-1 virus was not available. The plating efficiency of 9 strains including the five dengue-2 and the four dengue-4 strains could not be determined due to unsuccessful attempts to reisolate virus from original leukocyte suspensions employing LLC-Mk_2 cells. Dengue-3 virus was reisolated from each of two original human plasma specimens. As shown in Table 1, the PFU yield for both strains was greater at 32°C. Although the titer was slightly higher for strain #2878 the difference in plaque yield for each strain at 32°C and 35°C was comparable. The diameter of PFU for each strain at 32°C and 35°C ranged from 1 to 2 mm.

The observation that the optimal temperature for replication of the dengue-3 strains was 32°C proved to be helpful for routine isolation and identification of dengue viruses. As shown in Table 2, passage of five virus isolates at 37°C in LLC-Mk_2 cells to increase titers for virus identification resulted in a complete loss of infectivity. Subsequently, the same 5 isolates were assayed in LLC-Mk_2 cells at 32°C employing the original specimens and/or different LLC-Mk_2 passage material as a source of virus. The results presented in Table 3 showed each virus isolate to attain peak titer after one or two passage in at 32°C. In contrast to results obtained at 37°C, the titers of each virus isolate at 32°C remained approximately the same regardless of the passage level. Virus isolate #2737 was of particular interest in that the number of PFUs increased from 108 per 0.3 ml at 37°C too to numerous to count (TNTC) at 32°C where as the plaque count remained TNTC for #2713. All five isolates were subsequently determined to be dengue 3 virus. The clinical histories of the patients from whom the viruses were isolated were not available.

The rate and magnitude of replication of 2 strains of dengue-4, and one each of dengue-1 and dengue-2 that originated from patients, grade I and grade II illness, were determined at 32°C, 35°C and 37°C. No apparent difference was noted for the replication of each virus at the different temperatures, except for dengue 2 virus that was detected 12 to 24 hours prior to the recovery of the other viruses and peak titers for dengue-1 was attained 36 to 48 hours later than dengue 2 and dengue 4 viruses.

This investigation will be continued to further define the relationship of temperature to the replication of selected strains of dengue viruses.

Table 1. Replication of dengue-3 viruses in LLC-Mk_2 cells at different temperature.*

Dengue-3 Strain	Temperature (°C)	Plaque forming units			
		Undiluted	1	2	3
2878	32	TNTC**	TNTC	TNTC	78
(Non-fatal case)		TNTC	TNTC	TNTC	81
	35	TNTC	TNTC	105	16
		TNTC	TNTC	80	20
2797	32	TNTC	66	07	00
(Fatal case)		TNTC	86	09	00
	35	55	10	00	00
		-	6	00	00

* Plaques were not observed in LLC-Mk_2 cells at 39°C.

** Too numerous to count.

Table 2. Replication of virus isolates at 37°C in LLC-Mk_2 cells.

Plasma Number	Number of Passages							
	P-0	P-1	P-2	P-3	P-4	P-5	P-6	P-7
2737/77	108*	02	3,6	0				
2713/77	TNTC**	$80x10^3$	$9x10^2$	0				
2817/77	06	$1x10^2$	$3x10^3$	2x10	$2x10^1$	0		
2051/75***						$6x10^2$	$27x10^1$	0
H58525/77	4	$1x10^1$	$1x10^1$	0				

* Plaque forming units obtained by either direct or delayed plaque assay from original specimen.

** Too numerous to count.

*** Data prior to passage 5 was not available.

Table 3. Replication of virus isolates at 32°C in LLC-Mk_2 cells.

Plasma Number	Number of Passages							
	P-0	P-1	P-2	P-3	P-4	P-5	P-6	P-7
2737/77	TNTC	$46x10^3$						
2713/77	TNTC	$11x10^1$	$87x10^2$	$50x10^3$				
2817/77	–*			$84x10^1$	$9x10^3$	$2x10^3$		
2051/75**	–*						$7x10^1$	$5x10^2$
H58525/77	–*			$83x10^1$	$4x10^3$	$1x10^3$	$1x10^3$	$132x10^3$

* Original of these isolates were not available.

** Isolate number 2051 yielded $8x10^2$, $11x10^3$, $42x10^3$ at LLC-Mk_2 passage 8, 9 and 10, respectively.

REFERENCES :

1. Russell, P,K. and Nisalak, A. Dengue Virus Identification by the Plaque Reduction Neutralization Test. J. IMMUNOL., 99: 291-296, 1967.

2. Johnson, K.M., Halstead, S.B., and Cohen, S.N. Hemorrhagic fevers of Southeast Asia and South America. A comparative appraisal. PROG. MED. VIROL. 9: 105-158, 1967.

3. Hammon, W. McD. Immunological Response : Possible Role of Human Response as an Etiological Factor. Bull. Wld. Hlth. Org., 35: 55-56, 1966.

4. Halstead, S.B. and Simasthien, P. Observations Related to the Pathogenesis of Dengue Hemorrhagic Fever. II. Antigenic and Biologic Properties of Dengue Viruses and Their Association with Disease Response in the Host. Yale J. of Biol. & Med., 42: 276-292, 1970.

5. Halstead, S.B., Nimmannitya, S., and Cohen, S.N. Observations Related to Pathogenesis of Dengue Hemorrhagic Fever. IV. Relation of Disease Severity to Antibody Response and Virus Recovered. Yale J. Biol. Med., 42: 311-328, 1970.

6. Eckels, K.H., Brandt, W.E., Harrison, V.R., McCowan, J.M. and Russell, P.K. Isolation of Temperature-sensitive Dengue-2 Virus under Conditions Suitable for Vaccine Development. Infection and Immunity, 14: 1221-1227, 1976.