Evaluation of the C6/36 *Aedes albopictus Cell Line* as a Substrate for Dengue Virus Growth

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OBJECTIVE : To evaluate the utility of the C6/36 *Aedes albopictus* cell line as a substrate for dengue virus growth.

BACKGROUND : Difficulty is often encountered in attempting to raise infectivity titers of newly isolated dengue virus strains by repeated passage in either LLC-Mk2 cells or suckling mouse brain (SMB). Recently, a new mosquito cell line, the C6/36 *Aedes albopictus* cell line (1), was developed with the express goal of increased sensitivity and yield specifically for dengue viruses. This line was derived by cloning *A. albopictus* (Singh) cells in media containing anti-Chikungunya antibody in order to circumvent problems with chronic chikungunya virus infection encountered in other mosquito cell lines. A clone of cells (clone 6) which produced the highest yield of dengue viruses compared to other clones was then selected; this clone was then recloned and the most sensitive sub-clone (C6/36) selected to start the line. The C6/36 cell line is reported to show a cytopathic response to dengue virus infection, and may permit virus yields sufficiently high to allow for virus identification by the plaque reduction neutralization (PRNT) method. We elected to test the line using suckling mouse brain seeds of prototype dengue-1, -2, -3, and -4 viruses. To investigate if the line was also sensitive to virus with passage histories other than suckling mouse brain, we also tested a D2 mosquito passage seed and a dengue type 2 LLC-Mk2 tissue culture seed.

METHODS

Viruses: The following virus strains were tested for growth in C6/36 cells : Dengue virus type 2 (Den-2) New Guinea B strain at 29th passage in suckling mouse brain; Den-2 local strain (24742-74) isolated from the plasma of a dengue hemorrhagic fever patient (1974), at the 10th passage in LLC-Mk2 cells; Den-2 local strain passed once in *Toxorhynchites* mosquitoes; Den-1 Hawaiian strain at the 15th passage in suckling mouse brain (SMB); Den-3 H-87 strain at the 25th passage in SMB; Den-4 H-241 strain at 31st passage in SMB.

Cells : C6/36 cells at the sixth passage level were obtained from Dr. Nathirat Sankavipa at the Virus Research Institute in Bangkok. C6/36 cells were grown in RPMI 1640 with 10% inactivated fetal calf serum at 28°C. LLC-Mk2 cells were grown in Medium-199 with 15% calf serum at 35°C.

Virus growth curves : Confluent cell monolayers in one ounce bottles were inoculated with 0.1 ml aliquots of selected dilutions of each virus, and then incubated for 2 hours. The cultures were then replenished with 3 ml for RPMI

1640 with 5% inactivated fetal calf serum and placed at 35°C for LLC-Mk2 and 28°C for C6/36 cell cultures. Half of the medium was removed on each sampling day and replaced with new medium. The withdrawn fluid was titrated for infectivity and HA activity.

LLC-Mk2 cultures and C6/36 cultures were subjected to a freeze and thaw cycle in 3 ml of media on day 7 and 15, respectively, and the resultant suspension were then assayed for intracellular virus.

Assays for infectivity titer were done on LLC-Mk2 cells in microcultures in 24 well plastic micro-culture plates; HA activity was assayed, without extraction, by standard methods.

RESULTS : Simultaneous titration of the prototype D2 SM seed (D2NGC SM29) on LLC-Mk2 cells and C6/36 cells showed that both the sensitivity and yield of the C6/36 cells were superior for this virus (Table 1). A maximum yield of 10^8 PFU/ml on LLC-Mk2 cells. In addition, an HA titer of up to 1:256 per 0.025 ml was present in the tissue culture supernatant fluid from the C6/36 cells. Similarly good yields, sensitivity, and HA production were also obtained with the LLC-Mk2 passed virus D2 strain and the mosquito passed D2 strain (Table 1) At similar inoculum doses of approximately 10^3 PFU/0.2 ml, all three strains gave peak yields of 10^6-10^7 PFU/ml and peak HAI titers of 1:64 to 1:128.

The C6/36 cell line also supported the growth of D1, D3, and D4 suckling mouse brain seeds; however, peak infectivity titers were lower than with we found with D2 and also lower than previously reported by other workers for D1, D3, and D4 (1). Tables IIA, B, C). Peak HA titers found were 1:128 and 1:64 for D1 and D4 respectively; no HA activity was detected in the cell culture fluid from C6/36 cells inoculated with the D3 SMB seed.

REFERENCES :

1. Igarashi, A. Isolation of a Singh's *Aedes albopictus* Cell Clone Sensitive to Dengue and Chikungunya Viruses. J. Gen. Virol. 40:531-44, 1978.

Table I. Yields of LLC-Mk2 Cell Cultures and C6/36 Cell Cultures Inoculated with Dengue 2 Virus Seeds (SMB, LLC-Mk2, or Mosquito).

Virus Strain	Inoculum (PFU/0.2 ml)	Day	LLC-Mk2 Yield PFU/0.2 ml	LLC-Mk2 Yield HA	C6/36 Yield PFU/0.2 ml	C6/36 Yield HA
D2SMB	10^5	7	$3 \times 10^{4*}$	0	4.8×10^6	128
		11	-	-	4.5×10^6	256
		15	-	-	$1.2 \times 10^{6*}$	0
	10^4	7	$1.2 \times 10^{3*}$	0	1.3×10^6	32
		11	-	-	2.1×10^7	128
		15	-	-	$6 \times 10^{5*}$	0
	10^3	7	3×10^3	0	3×10^5	0
		11	-	-	2.1×10^6	128
		15	-	-	$1.3 \times 10^{6*}$	0
	10^2	7	$3 \times 10^{4*}$	0	9×10^5	0
		11	-	-	1.2×10^6	16
		15	-	-	$7.5 \times 10^{5*}$	0
	10	7	$8 \times 10^{3*}$	0	1.2×10^3	0
		11	-	-	1.5×10^5	0
		13	-	-	5.7×10^4	16
		15	-	-	$6 \times 10^{3*}$	0
	1	7	$6 \times 10^{3*}$	0	1.8×10^2	0
		15	-	-	$4.8 \times 10^{3*}$	0
	10^{-1}	7	0	0	4.5	0
		15	-	-	$6 \times 10^{2*}$	0
D2-LLC-Mk2	5×10^3	7	$4 \times 10^{4*}$	0	3×10^5	128
		11	-	-	3×10^5	128
		15	-	-	$9 \times 10^{6*}$	0
	5×10^2	7	$5.5 \times 10^{4*}$	0	3×10^4	0
		11	-	-	7.5×10^5	32
		15	-	-	$5.1 \times 10^{6*}$	0
	5×10	7	$8 \times 10^{4*}$	0	4.5×10^5	16
		11	-	-	3×10^5	32
		15	-	-	$4.3 \times 10^{6*}$	0

Virus Strain	Inoculum (PFU/0.2 ml)	Day	LLC-Mk2 Yield		C6/36 Yield	
			PFU/0.2 ml	HA	PFU/0.2 ml	HA
D2-LLC-Mk2	5	7	6×10^4	0	6×10^2	0
		11	-	-	2.7×10^6	32
		15	-	-	$3.9 \times 10^{5*}$	0
	5×10^{-1}	7	$2 \times 10^{4*}$	0	3×10^2	8
		11	-	-	3×10^5	0
		15	-	-	$9 \times 10^{6*}$	0
	5×10^{-2}	7	$6 \times 10^{2*}$	0	1^2	
		11	-	-	10^2	0
		15	-	-	10^{2*}	0
	5×10^{-3}	7	0*	0	0	0
		11	-	-	0	0
		15	-	-	10^{2*}	0
D2-Mosq.	4×10^3	7	ND		8.7×10^6	64
		11			4.5×10^3	0
		15			3×10^4	0
	4×10^2	7			3.6×10^6	128
		9			6×10^5	0
		13			4.5×10	0
		15			Contam	
	4×10	7			1.5×10^4	0
		9			6×10^4	0
		13			7.5×10	0
		15			Contam	
	4	7			1.5×10^4	0
		9			1.2×10^4	0
		11			9×10^2	0
		15			$1.5 \times 10^{5*}$	0
	4×10^{-1}	7			$1.5 \times 10^{-3}_2$	0
		11			2.4×10^2	0
		15			$6 \times 10^{2*}$	0
	4×10^{-2}	7			0	0
		11			0	0
		15			0	0

* = After one freeze - thaw cycle of cells.

Table IIA. Yield of C6/36 Cell Cultures Inoculated with D1 SMB Seed.

Virus Strain	Inoculum PFU/0.2 ml	Day	C6/36 Yield PFU/0.2 ml	C6/36 Yield HA
D1SM	2 x 10	7	5×10^4	64
		9	1×10^4	128
		12	3×10^3	64
		15	2×10^4	0
	2×10^3	7	5×10^4	64
		9	1×10^4	128
		12	6×10^3	64
		15	$1 \times 10^{4*}$	0
	2×10^2	7	2×10^4	32
		9	3×10^4	128
		12	2×10^4	32
		15	Contam	
	2 x 10	7	1×10^4	32
		9	1×10^4	64
		12	1×10^4	32
		15	1×10^3	0
	2	7	2×10^3	0
		9	2×10^4	0
		12	1×10^3	0
		15	5×10^3	0
	2×10^{-1}	7	10	0
		9	2×10^2	0
		12	1×10^2	0
		15	1×10^3	0
	2×10^{-2}	7	0	0
		9	5×10	0
		12	1×10^2	0
		15	8×10	0

Table IIB. Yield of C6/36 Cell Cultures Inoculated with D3 SMB Seed.

Virus Strain	Inoculum PFU/0.2 ml	Day	C6/36 Yield PFU/0.2 ml	C6/36 Yield HA
D3SM	2×10^3	7	1×10^4	0
		9	2.5×10^4	0
		12	5×10^3	0
		15	Contam	0
	2×10^2	7	2×10^4	0
		9	2.5×10^4	0
		12	5×10^3	0
		15	Contam	
	2×10	7	6×10^3	0
		9	1×10^3	0
		12	1.6×10^2	0
		15	Contam	0
	2	7	3×10^4	0
		9	5×10^4	0
		12	2×10^3	0
		15	Contam	
	2×10^{-1}	7	2×10^4	0
		9	8×10^4	0
		12	6×10^3	0
		15	Contam	
	2×10^{-2}	7	1.4×10^4	0
		9	5×10^4	0
		12	1×10^3	0
		15	Contam	
	2×10^{-3}	7	4×10^2	0
		9	1×10^2	0
		12	6.8×10^2	0
		15	Contam	

Table IIC. Yield of C6/36 Cell Cultures Inoculated with D4 SMB Seed.

Virus Strain	Inoculum PFU/0.2 ml	Day	C6/36 Yield PFU/0.2 ml	C6/36 Yield HA
D4 SM	4×10^6	7	3×10^4	8
		9	3×10^4	8
		11	3×10^2	0
	4×10^5	7	5×10^4	8
		9	3×10^5	64
		11	1.8×10^4	0
	4×10^4	7	4×10^4	0
		9	4×10^5	64
		11	6×10^4	0
	4×10^3	7	1.3×10^4	0
		9	5×10^4	0
		11	6×10^4	0
	4×10^2	7	6×10^3	0
		9	3×10^4	0
		11	1.8×10^4	0
	4×10	7	6×10^2	0
		9	1.2×10^4	0
		11	8×10^3	0
	4	7	5×10^2	0
		9	1.6×10^3	0
		11	6×10	0
	4×10^{-1}	7	4.5×10	0
		9	2×10^2	0
		11	1×10^2	0
	4×10^{-2}	7	0	0
		9	0	0
		11	0	0