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REPLICATION OF DENGUE, YELLOW FEVER, ST. LOUIS ENCEPHALITIS AND VESICULAR STOMATITIS VIRUSES IN A CELL LINE (TRA-171) DERIVED FROM *TOXORHYNCHITES AMBOINENSIS*

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SUMMARY

The replication of seven arboviruses in a cell line (TRA-171) derived from a nonhematophagous mosquito was studied. Four serotypes of laboratory adapted and three serotypes of unadapted dengue viruses replicated in the TRA-171 cell line, inducing syncytia. The sensitivity of TRA-171 cells to dengue virus infection was comparable to that of *Aedes albopictus* or *A. pseudoscutellaris* cells. Yellow fever, St. Louis encephalitis, and vesicular stomatitis viruses also replicated. All four serotypes of dengue viruses could be plaque assayed with TRA-171 cell cultures.

Key words: mosquito cell; arbovirus; cytopathic effect.

INTRODUCTION

In the past, arbovirus replication has been studied primarily either with mammalian cells or mosquito cells derived from hematophagous mosquitoes. Although the adults of *Toxorhynchites amboinensis* are not vectors of viruses, they serve as excellent artificial hosts for the propagation of dengue viruses by means of intrathoracic inoculation (1). At least two cell lines had been isolated from this nonhematophagous mosquito but did not prove to be significantly sensitive to dengue viruses (2). Inasmuch as another cell line was independently isolated from *T. amboinensis* (3), it was of interest to study the replication of dengue viruses in that cell line and compare the sensitivity to the viral infection with that of two hematophagous mosquito cell lines known to be sensitive to dengue virus infection. Furthermore, the replication of St. Louis encephalitis, yellow fever, and vesicular stomatitis viruses was studied in the *T. amboinensis* cell cultures.

MATERIALS AND METHODS

Cell cultures. The *Toxorhynchites amboinensis* cell line (TRA-171) was maintained at 28° C with MM/VP medium (4) without antibiotics in 25 cm^2 plastic tissue culture flasks. Cells were used at the 20 to 24 passage levels.

Singh's *Aedes albopictus* cells (Clone C6/36) (5) were obtained from Dr. R. B. Tesh (Yale University, New Haven, CT) and grown in Eagle's minimum essential medium (MEM) containing 0.2 m*M* nonessential amino acids and 10% heat-inactivated fetal bovine serum (FBS) (5). The cells were used at passage levels above 50.

Aedes pseudoscutellaris cells (AP-61) (6) were grown in MM/VP 12 medium without antibiotics and used at the 46th passage.

Viruses. Before inoculation, four serotypes of laboratory adapted dengue viruses (DEN), yellow fever virus (YF), St. Louis encephalitis virus (SLE), and vesicular stomatitis virus (VSV) were diluted in a phosphate buffered saline (PBS) (pH 7.3) containing 20% heat-inactivated FBS (1 h at 56° C). The strain and passage history of each virus are as follows:

Adapted dengue viruses: DEN-1 (Hawaii) 1 monkey, 1 mosquito, and 17 tissue culture passages; DEN-2 (NG"C") 24 suckling mouse and 5 tissue culture passages; DEN-3 (PR-6) 13 suckling mouse passages; and DEN-4 (H-241) 7 suckling mouse and 6 tissue culture passages.

Unadapted dengue viruses: sixteen human sera from which dengue viruses (Types 1, 2, and 3) had been isolated by intrathoracic inoculation of adult mosquitoes: YF (17D) suckling mouse and two tissue culture passages; SLE (P-15) four suckling mice and three tissue culture passages; and VSV (New Jersey) more than two tissue culture passages.

Inoculation. Four serotypes of adapted dengue viruses and sixteen strains of unadapted dengue viruses were simultaneously inoculated in aliquants of 0.2 ml/flask into flask cultures of three cell lines. Yellow fever, SLE, and VSV were inoculated similarly only into TRA-171 cells. After 90 min virus adsorption at 28° C, the flasks were rinsed once with PBS and incubated at 28° C with maintenance media. For *A. albopictus* cells the maintenance medium was Eagle's MEM containing 2% FBS; for AP-61 cells it was Leibovitz (L15) medium containing 10% tryptose phosphate broth and 5% glutamine (200 m*M*); and for TRA-171 cells it was a mixture of equal volumes of MM/VP 12 medium and L15 medium.

Virus titration. Aliquants (0.5 ml) of supernatant fluids were withdrawn from the inoculated flasks and replaced daily with equal volumes of fresh maintenance media for the first 5 d and on Days 7, 9, and 12 after inoculation. Virus titers of the inocula as well as the harvested supernatant fluids were plaque assayed in LLC-MK_2 cell cultures, according to the method of Eckels et al. (7).

Plaquing viruses in TRA-171 cells. Tenfold serial dilutions of each virus were inoculated in aliquants of 0.2 ml/flask into a monolayer culture of TRA-171 cells. After 90 min virus adsorption, the flasks were overlaid with 6 ml of the first plaquing medium containing 0.75% agarose (Seaplaque brand, Marine Colloids, Inc., Rockland, ME), 0.25% purified agar (Difco, Detroit, MI), 10% FBS (heat inactivated at 56° C for 1 h), 1.5% essential amino acids (100X), 1.5% vitamin mixture (100X), 1.4% glutamine (200 m*M*), and 0.1% sodium bicarbonate (7.5%) in L15 medium without phenol red. After 7 d incubation at 32° C, the flasks were overlaid with 4 ml of the second plaquing medium containing 7 ml neutral red (1:300)/100 ml medium. The flasks were incubated at 35° C for 1 to 2 d before the plaques were counted.

RESULTS

Replication of laboratory adapted dengue viruses. The results in Table 1 show that all four serotypes of adapted dengue viruses replicated in TRA-171 cells as well as in the other two cell lines. The extracellular virus titers generally rose earlier in *A. albopictus* cells than in AP-61 or TRA-171 cells. Although the doses of four serotypes used were not exactly the same, it was apparent that DEN-3 (PR-6) replicated more slowly

TABLE 1

REPLICATION OF LABORATORY ADAPTED DENGUE VIRUSES IN THREE MOSQUITO CELL LINES

Virus	Multiplicity of Infection (PFU/cell)	Cell Line	Extracellular Virus Titer, Days After Inoculation: 0	1	2	3	4	5	7
			log_{10} PFU/ml supernatant fluid						
DEN 1		AAL[a]	—[b]	2.18	3.18	4.90	5.18	5.40	7.53
(Hawaii)	0.0006	APS[c]	—	—	3.08	1.70	2.90	4.82	6.90
		TRA[d]	—	—	2.60	4.00	5.92	4.30	6.37
DEN 2		AAL	—	—	2.48	4.00	4.70	5.20	7.15
(NG"C")	0.0002	APS	—	—	2.18	2.18	2.65	3.70	6.45
		TRA	—	—	—	3.91	4.40	3.78	6.00
DEN 3		AAL	—	—	2.00	—	2.00	3.88	5.00
(PR-6)	0.001	APS	—	—	—	—	—	3.43	5.70
		TRA	—	—	—	—	2.43	5.13	4.70
DEN 4		AAL	—	3.04	3.30	4.54	5.30	6.40	7.58
(H-241)	0.008	APS	—	—	2.70	—	3.11	4.70	7.62
		TRA	—	—	—	2.70	2.00	5.15	6.28

[a] AAL: *Aedes albopictus* cells (Clone C6/36 by Igarashi).
[b] No detectable titer.
[c] APS: *Aedes pseudoscutellaris* cells by Varma et al.
[d] TRA: *Toxorhynchites amboinensis* cells (TRA-171) by Kuno.

in all three cell lines than the other serotypes. Syncytia developed in AP-61 cells and TRA-171 cells inoculated with all serotypes, most typically 5 to 10 d after inoculation. Syncytia developed in low frequency also in normal cell controls of TRA-171 at passage levels below 30 but not above 40. These syncytia in normal cell controls remained small in size and could be distinguished from virus-induced syncytia, which progressively grew larger to cover the entire flask surface in 12 d. Furthermore, no virus was found in the TRA-171 cells that developed "spontaneous" syncytia. No cytopathic effect (CPE) was observed in *A. albopictus* cells incubated at 28° C despite viral replication.

Replication of unadapted dengue viruses. Serotypes 1, 2, and 3 of unadapted dengue viruses replicated in the three cell lines. Out of 16 sera

TABLE 2

REPLICATION OF UNADAPTED DENGUE VIRUSES IN THREE MOSQUITO CELL LINES

Virus Serotype	Specimen No.	Inoculum (PFU/Flask)	Cell Line	Extracellular Virus Titer, Days After Inoculation 0	1	2	3	4	5	7	9
				log PFU/ml supernatant fluid							
DEN 1	1	6	AAL[a]	—[b]	—	—	—	—	3.88	4.83	4.92
			APS[c]	—	—	—	—	—	—	—	4.92
			TRA[d]	—	—	—	—	—	3.05	2.00	3.36
	2	7.8×10^3	AAL	—	2.00	2.00	4.47	4.95	5.28	5.78	5.43
			APS	—	—	—	2.00	3.08	3.30	6.45	6.26
			TRA	—	—	1.38	4.79	4.59	4.51	4.32	4.79
	3	32	AAL	—	—	—	—	—	—	4.15	5.96
			APS	—	—	—	—	—	3.23	2.48	4.48
			TRA	—	—	—	—	2.60	3.60	4.56	5.70
	4	0	AAL	—	—	—	—	—	—	—	—
			APS	—	—	—	—	—	—	—	—
			TRA	—	—	—	—	—	—	—	4.17
DEN 2	5	68	AAL	—	—	—	2.65	3.32	3.40	3.95	4.63
			APS	—	—	—	—	—	3.68	2.48	4.48
			TRA	—	—	—	1.70	2.00	3.94	4.56	4.71
	6	16	AAL	—	—	—	2.48	3.54	3.98	5.86	5.05
			APS	—	—	—	—	—	—	5.10	5.12
			TRA	—	—	—	1.70	2.40	4.20	4.04	5.07
	7	167	AAL	—	—	—	—	1.70	—	2.70	2.70
			APS	—	—	—	—	—	—	2.48	3.60
			TRA	—	—	—	—	—	1.70	—	3.00
	8	1	AAL	—	—	1.70	1.70	3.00	—	4.81	6.24
			APS	—	—	—	—	—	—	6.09	6.73
			TRA	—	—	—	—	—	2.00	5.18	4.60
DEN 3	9	10	AAL	—	—	—	—	—	—	4.00	4.70
			APS	—	—	—	—	—	—	—	3.65
			TRA	—	—	—	—	—	—	—	3.18
	10	23	AAL	—	—	—	—	—	—	3.70	2.70
			APS	—	—	—	—	—	—	2.70	5.18
			TRA	—	—	—	—	—	—	2.00	4.17
	11	22	AAL	—	—	—	2.40	3.08	2.90	3.81	4.13
			APS	—	—	—	—	—	—	—	4.23
			TRA	—	—	—	—	—	2.65	3.00	5.03
	12	5	AAL	—	—	—	—	—	2.00	3.95	5.97
			APS	—	—	—	—	—	—	—	3.90
			TRA	—	—	—	—	—	—	1.30	3.54

[a] AAL: *Aedes albopictus* cells (C6/36) by Igarashi.
[b] No detectable titer.
[c] APS: *Aedes pseudoscutellaris* cells by Varma et al.
[d] TRA: *Toxorhynchites amboinensis* cells (TRA-171) by Kuno.

tested, the results obtained with 4 sera of each serotype are demonstrated in Table 2. With respect to unadapted DEN-2 and DEN-3 viruses, titers often rose earlier in *A. albopictus* cells than in the other two cell lines. Although virus titers remained at the same levels in TRA-171 or AP-61 cells after 9 through 12 d postinoculation, they dropped in *A. albopictus* cells. In terms of the highest virus titer obtained, the three cell lines were more or less comparable. In addition, in terms of virus isolation rate, the three cell lines were found to have similar sensitivity, inasmuch as 16, 14, and 15 virus strains were isolated in TRA-171, *A. albopictus*, and AP-61 cells, respectively. Syncytia developed in TRA-171 and AP-61 cell cultures from which viruses were isolated; no syncytia developed in *A. albopictus* cells.

Replication of YF, SLE, and VSV. The results in Table 3 show that all three viruses replicated in TRA-171 cells. The highest extracellular virus titers for VSV, YF, and SLE were obtained 2, 3, and 5 d after inoculation, respectively. No cytopathic effect was observed.

Plaque development. Plaques of four serotypes generally were larger in TRA-171 cells than those in LLC-MK_2 cells. However, virus titers [plaque-forming unit (PFU)] in TRA-171 cells were approximately 10 times lower than those in LLC-MK_2 cells. No plaques of YF, SLE, or VSV were obtained.

Discussion

That cell lines from a nonhematophagous mosquito, *T. amboinensis*, could support replication of some arboviruses was reported previously (2). In contrast to the previously isolated cell lines (2), however, TRA-171 cells demonstrated a sensitivity to dengue virus infection comparable to that of *A. albopictus* or AP-61 cells. Daily monitoring of extracellular virus titer in cell cultures inoculated with unadapted viruses provides important information regarding the optimum length of incubation period necessary for the virus recovery. For dengue virus identification by the plaque reduction neutralization test (PRNT) (8), the minimum concentration necessary for obtaining 50% plaque reduction end point is approximately 10^3 PFU/ml, assuming 0.1 ml each of virus and antidengue immune serum are mixed and inoculated into a flask. The results (Table 2) demonstrated that for the majority of specimens, 7 to 9 d incubation was sufficient in all three cell lines unless the inocula were extremely low titered. Dengue viruses can be identified faster by complement fixation test (CF) than by PRNT with the use of CF antigen in infected mosquito cell culture (1). However, CF antigens in mosquito cultures are often low titered (1) or anticomplementary.

Although several hematophagous mosquito cell lines are known to develop syncytia upon dengue virus infection (5,6,9), no cell line from nonhematophagous mosquitoes has been shown to develop CPE by dengue viruses. Therefore, this is the first report of syncytial induction by dengue viruses in a nonhematophagous cell line. There is a strong possibility that spontaneous syncytial development in normal controls is a function of low level of cellular adaptation in vitro, since it was not seen in TRA-171 cells at passage levels beyond 40. With TRA-171 cells at higher passage levels, the syncytial development became a more reliable indicator of dengue virus infection. Syncytial development in *A. albopictus* cell line by dengue viruses has been documented. However, in this study, syncytia failed to develop in *A.* albopictus cells inoculated with dengue viruses and maintained at 28° C, which contradicted the past report (5). Several factors regulating the induction of syncytia in *A. albopictus* cells by an arbovirus were reported elsewhere (10). Further efforts to induce syncytia in the cloned cell line according to the method of Igarashi (5) were successful with or without 5% CO_2 only when the

TABLE 3

Replication of Yellow Fever, St. Louis Encephalitis, and Vesicular Stomatitits Viruses in a Cell Line From *Toxorhynchites amboinensis*, TRA 171

Virus (Strain)	Multiplicity of Infection	Extracellular Virus Titer, Days After Inoculation					
		1	2	3	4	5	7
	PFU per cell	*log PFU/ml supernatant fluid*					
YF (17-D)	57×10^{-5}	2.00	3.00	4.48	4.00	3.00	3.78
SLE (P-15)	7×10^{-5}	NDT	3.00	4.30	4.85	6.28	5.18
VSV (New Jersey)	13×10^{-5}	3.30	6.48	5.00	4.88	3.93	3.81

NDT = No detectable titer by plaque assay using LLC-MK_2 cells.

cells were inoculated with DEN-2 (NG C) and incubated at 32° C. Although none of the unadapted dengue viruses used during the experiment induced syncytia in *A. albopictus* cells, in another experiment syncytia were found to be induced at 28° C by one unadapted dengue virus out of 47 unadapted strains, all of which induced syncytia in TRA-171 and AP-61 cells. The results, therefore, indicated that syncytium was a more useful indicator of dengue virus infection in TRA-171 or AP-61 cells than in *A. albopictus* (Clone C6 36) cells.

The fact that SLE, VSV, and YF replicated in TRA-171 cells suggests a possibility that many other arboviruses not used in this test also replicate in the cell line. In fact, Tesh (2) demonstrated that his cell lines from *T. amboinensis* were sensitive to several viruses not included in this study.

Yunker and Cory (11) described plaque development of many arboviruses including DEN, YF, SLE, and VSV in *A. albopictus* cells. Plaque development by DEN-1, DEN-2, and unadapted strains of YF also were reported with AP-61 cells by Pudney et al. (9). On the other hand, only four serotypes of dengue viruses produced plaques in TRA-171 cells. It was not ascertained if the failure of plaque development by YF, SLE, or VSV in TRA-171 cells was a characteristic of the cell line or of inadequate plaquing medium, or plaquing conditions, or both.

The above results demonstrated that replication of some arboviruses can be studied in vitro by using a cell line derived from a nonhematophagous mosquito and suggested that TRA-171 may be a useful tool for the isolation of dengue viruses from human sera.

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