A NEW CELL LINE FROM THE EMBRYONIC TISSUES OF *CULEX TRITAENIORHYNCHUS* AND ITS SUSCEPTIBILITY TO CERTAIN FLAVIVIRUSES

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Summary. – A new cell line from the embryonic tissue of *Culex tritaeniorhynchus* mosquito was established. Morphological studies carried out at the 45th passage level (P-45) showed four different cell types *viz.* epithelial-like cells, fibroblast-like cells, giant cells and vacuolated cells. Karyological studies indicated diploid (2n = 6) chromosomes in majority of cells irrespective of passage level. A twelve-fold increase of cell number was observed in 10 days at P-49. The cells could be preserved in liquid nitrogen for more than 40 months. Isoenzyme profile analysis with four enzymes clearly indicated that this cell line was derived from *C. tritaeniorhynchus*. This cell line was susceptible to Japanese encephalitis (JEV) and West Nile viruses (WNV) but not to Dengue 1-4 (DEN 1-4) viruses. Protein of 38 K was detected in the membrane fraction of the cells from this and the C6/36 cell line, which was found to bind DEN 1-4 viruses. These data suggest that DEN viruses bind to this membrane protein and probably enter into the cells but do not continue further in the replication process.

Key words: cell membrane protein; Culex tritaeniorhynchus; Dengue viruses; isoenzyme; Japanese encephalitis virus; mosquito cell line; virus susceptibility; West Nile virus

Introduction

A large number of mosquito cell lines have been established since 1967 and several of them are effectively used for the isolation of arboviruses (Pant *et al.*, 1985). Cell lines from *Aedes* mosquitoes are available, which are very useful for isolation of DEN viruses, however only a few cell lines are available from *Culex* mosquitoes. In an attempt to establish further flavivirus-susceptible cell lines from *Culex* mosquitoes to facilitate virus isolation, a new cell line from embryonic tissues of *C. tritaeniorhynchus* mosquitoes was established. The new cell line is an addition to the mosquito cell lines existing in the world. Its high susceptibility to certain flaviviruses might have advantage in their isolation. The present communication reports the establishment and characterization of this cell line inclusive of its susceptibility to certain arboviruses.

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Abbreviations: BSA = bovine serum albumin; DEN = Dengue virus; FBS = fetal bovine serum; IIFT = indirect immunofluorescent technique; JEV = Japanese encephalitis virus; MM medium = Mitsuhashi-Maramorosch medium; PBS = phosphate-buffered saline; p.i = post infection; PMSF = phenylmethanesulfonyl fluoride; WNV = West Nile virus

Materials and Methods

Establishment of the cell line. The mosquito eggs used to set up the primary cultures were obtained from our laboratory breeding. The eggs were surface-sterilized and primary stationary cultures were prepared as described by Pant and Dhanda (1982). Thirty to forty eggs were used to set up one primary culture. As growth medium the Mitsuhashi Maramorosch medium (MM medium) (Mitsuhashi and Maramorosch, 1964) with 20% of fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin and 40 μg/ml gentamycin was used. The cultures were incubated at 28°C; 50% of the medium was replaced by a fresh one every 7–10 days until monolayers were formed.

Table 1. Characteristics of viruses used in the study

	Virus	Strain	Passage level	Titer of virus suspension (log ID_{50} per 0.2 ml)[a]	Virus inoculum (ID_{50} per 0.1 ml)[b]	Virus susceptibility status[c]
1	JEV	P-20778	16	6.2	7.9×10^5	+
2	JEV	753101	15	6.0	5.0×10^5	+
3	WNV	E-101	19	4.2	7.9×10^3	+
4	DEN 1	Hawai	16	3.1	6.3×10^2	–
5	DEN 2	TR-1751	12	3.8	3.2×10^3	–
6	DEN 3	633798	12	4.4	1.3×10^4	–
7	DEN 4	642069	32	4.8	3.2×10^4	–

[a]Estimated on mice by intracerebral inoculation.
[b]Used for infection of cells in virus susceptibility studies.
[c](+) = susceptible; (–) = unsusceptible.

Subcultivation. Only cultures with confluent monolayers were used for subcultivation. The cells were gently dispersed into a medium using a Pasteur pipette and the cell suspension was distributed equally in two new bottles each containing fresh medium. Initially, for comparison, the spent medium and the fresh growth medium were used for dispersing the cells. However, after 10 passages, when it was clear that there was no difference between their effects on the cell growth, only the fresh medium was used. Subcultivation was performed every 5–7 days based on the cell growth.

Morphological and karyological studies were performed on monolayer cultures grown on coverslips in Leighton tubes and stained with Giemsa. (Paul, 1970). The morphology of the cells was studied under a phase contrast microscope. At least 1000 cells were counted and the percentage of different cell types was determined. For karyological studies the cell preparations were observed under a microscope with oil immersion (magnification 100x). At least 100 chromosome spreads were counted to determine the ploidy of the cell line at different passage levels.

Growth curve studies. The cells were seeded in Belco tubes in the growth medium. Two tubes per day were selected randomly for 10 days and viable cell counts were made. Means from four counts were used for determination of the cell growth rate.

Isoenzyme studies. Four isoenzymes *viz.* lactate dehydrogenase, malate dehydrogenase, glucose-6-phosphate dehydrogenase and isocitrate dehydrogenase were followed for determination of species specificity of the cell line (Tabachnick and Knudson, 1980). Besides the new cell line, the C6/36 line and the cells from *C. bitaeniorhynchus* and *C. tritaeniorhynchus* mosquitoes were also analyzed for comparison.

Virus susceptibility studies. JEV, WNV and DEN 1-4 viruses were used to determine virus susceptibility of the new cell line. Details of virus strains used in this study is given in Table 1. All these strains, passaged in the mouse brain, were obtained from the Virus Collection of the National Institute of Virology, Pune, India. The susceptibility of the new cell line to these viruses was determined at P-145. Confluent monolayer cultures grown on coverslips in Leighton tubes were used. Briefly, the spent medium was discarded and 0.1 ml of a virus inoculum was added. Mock-infected controls were inoculated with the growth medium. The cultures were incubated at 28°C for 1 hr with rocking at 10-mins intervals. The virus inoculum was discarded, the cells were washed once with phosphate-buffered saline (PBS), fed with a maintenance medium (the growth medium containing only 2% of FBS) and further incubated. The cells were fixed at various times post infection (p.i.), stained for the detection of virus antigen.

Virus detection. The virus antigen in the cells was detected by an indirect immunofluorescence antibody technique (IIFT) (Ilkal *et al.*, 1984).

Cell membrane fraction (MF) from the new cell line was prepared largely according to Nielsen-Leroux and Charles (1992). The cells were dislodged by pipetting and the resulting suspension was centrifuged at 3,000 x g for 5 mins. The pellet was washed twice with the buffer A (0.3 mol/l Mannitol, 10 mmol/l EDTA, 20 mmol/l Tris, 1 mmol/l PMSF pH 7.4). The pellets were pooled and ground in the ice-cold buffer B (0.3 mol/l Mannitol, 10 mmol/l EDTA, 20 mmol/l Tris, 0.1 mmol/l PMSF, 12 mmol/l $MgCl_2$, 0.2% Triton X-100 pH 7.4) using sterile pestles (Kontes, USA) and microfuge tubes. The suspension was then centrifuged at 3000 x g in a microfuge for 15 mins at 4°C and the supernatant was saved and kept on ice for 20 mins. The pellets were resuspended in the buffer B and the resulting suspensions were pooled and subjected to the second grinding as above. The supernatant obtained was pooled with the first one and centrifuged at 11,000 x g for 1 hr. The resulting pellet was then resuspended in the buffer A. The protein yield was determined by the method of Lowry *et al.* (1951).

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE). To prepare samples for SDS-PAGE the MFs were mixed with a boiling mixture consisting of 0.62 mol/l Tris-HCl pH 6.9 with 0.5 mol/l 2-mercaptoethenol (1 part) and 0.2 mol/l SDS (Sigma, 3 parts) and heated at 100°C for 5 mins. Five and ten % acrylamide was used in separation gels with a constant current of 6 mA for 1–2 hrs. Molecular size markers (Sigma) were included in each gel. The gels were silver-stained according to Kazuaki and Ebata (1983).

Western blot analysis. Non-stained SDS-PAGE gels were blotted onto nitrocellulose membranes. The assay was carried out according to Mourya *et al.* (1998). Briefly, blot strips were washed in PBS, blocked with 5% BSA and incubated with the respective semipurified viruses (0.6 ml in PBS) at 37°C for 2 hrs. After thorough washing they were allowed to react with anti-virus IgG raised in mice (0.005 mg/0.6 ml in PBS containing 1% BSA and

0.1% Tween-20) at 37°C for 2 hrs. The reaction was detected using anti-mice antibodies tagged with alkaline phosphatase and 5-bromo-4-chloro-3-indonyl phosphate as substrate (Sigma).

Results and Discussion

Growth rate studies of the cell line were carried out at P-49, which indicated a 12-fold increase in the cell number on the day 10 after seeding. A steady growth of cells was observed from the day 2 till the day 10 after seeding. The population doubling time was found to be approximately 48 hrs during the logarithmic phase.

Morphological studies carried out at P-45 indicated a heterogeneous population of cells (Fig. 1). The cell line consisted of four morphologically distinct cell types: epithelial-like cells (88%), fibroblast-like cells (8%), giant cells (3.6%) and vacuolated cells (0.4%).

Karyological studies carried out at P-45, P-109 and P-186 showed a diploid number of chromosomes (2n = 6) in 84%, 68% and 71% of cells, respectively. Tetraploidy was observed in 23% of the cells at advanced passage levels (P-109 and P-186). Heteroploid number of chromosomes was observed in a few cells irrespective of passage level. The chromosome complement consisted of three pairs of chromosomes, which were unequal in size (Fig. 2). There was neither any significant change in the ploidy nor any structural aberration in chromosomes even at a high passage level (P-186).

The cells at P-10, P-18 and P-48 were stored in liquid nitrogen using 10% dimethyl sulfoxide as cryopreservant. Revived cells attached quickly to the surface and a monolayer was formed within 48 hrs. The cells could be cryopreserved for 40 months with a revival rate of 100%.

The isoenzyme profile analysis using four enzymes indicated the origin of the cell line from *C. tritaeniorhynchus* mosquitoes (data not shown). There was a marked difference in the position of the enzyme profile of the new cell line as compared to other cell lines.

The cell line supported the growth of JEV and WNV but did not that of DEN viruses (Table 1). JEV antigen could be detected on the day 1 p.i. in a few cells, while a maximum number of cells (90%) were found infected on the day 5 p.i. onwards. Similarly, in the case of WNV, few cells were found infected on the day 1 p.i., while >80% cells were found positive on the day 7 p.i. It was interesting to note that this cell line supported the multiplication of JEV and WNV, while it did not that of DEN viruses despite they all belong to same family (*Flaviviridae*). Virus growth was not observed in any of the DEN virus-infected cultures up to the day 10 p.i. Cytopathic effect was not observed in the cells infected with JEV or WNV at any stage. DEN 1-4 viruses-containing tissue culture fluids collected on the day 10 p.i. were also inoculated into infant

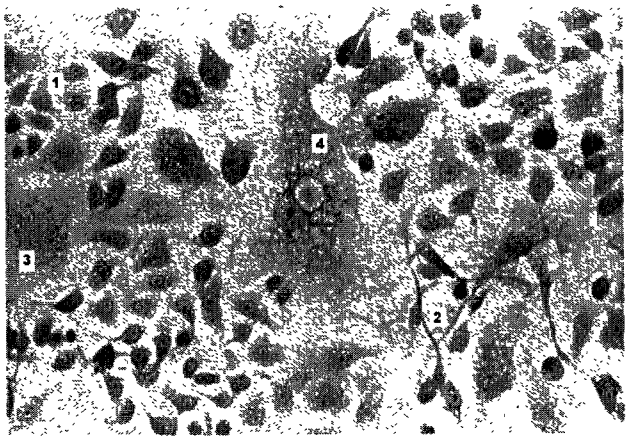

Fig. 1
The cell line at P-45 showing various cell types
Epithelial-like cells (1), fibroblast-like cells (2), giant cells (3), and vacuolized cells (4). Giemsa staining, magnification 400x.

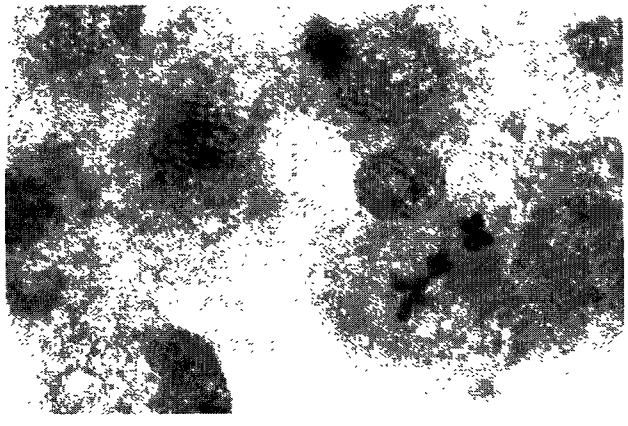

Fig. 2
The cell line showing cells with diploid chromosomes
Giemsa staining, magnification 1600x.

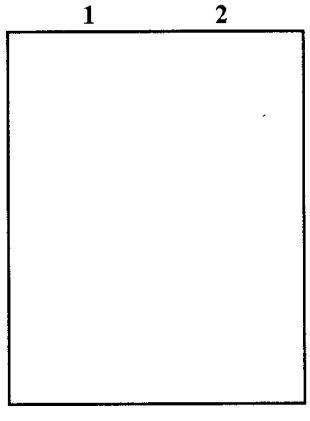

Fig. 3
Western blot analysis of Den virus-binding proteins from a membrane fraction of C6/36 (lane 1) and *C. tritaeniorhynchus* (lane 2) cell lines

mice but no sickness was observed. This further confirmed that this cell line is refractory to DEN viruses.

As this cell line is at present at P-202 it can be termed a continuous cell line. A steady growth of the cells was observed in the growth medium described above.

As *C. tritaeniorhynchus* mosquito species is the incriminated vector of JEV and WNV the multiplication of these viruses in this cell line was expected. The non-susceptibility of this cell line to DEN viruses, which belong to the same family (*Flaviviridae*) as do JEV and WNV, was not expected and is interesting.

The Western blot studies indicated that both the C6/36 and *C. tritaeniorhynchus* cell lines had putative virus binding proteins (Fig. 3). However, another *Culex* cell line developed from *C. bitaeniorhynchus* mosquitoes, which is also known to transmit JEV, supported the multiplication of not only JEV and WNV but also a DEN viruses (Pant *et al.*, 1985). There is experimental evidence suggesting that even after intrathoracic inoculation of DEN viruses into *C. quinquefasciatus* mosquitoes no growth of virus occurred (Vazeille-Falcoz *et al.*, 1999); this species is considered totally refractory to DEN virus transmission. However, cell lines developed from this species supported the multiplication of a DEN virus (Hsu, 1971). Two cell lines developed from the ovarian tissues of *C. quinquefasciatus* and *C. tritaeniorhynchus* have been shown to require different growth factors (Hsu, 1971).

Based on these data can be hardly generalized that a cell line developed from the mosquito species which is a natural vector of the given virus will support multiplication of that virus. The new cell line described here, due to its high susceptibility to JE and WN viruses, may prove useful for studies leading to a quick detection, isolation and characterization of these viruses.

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