ELECTRON MICROSCOPIC EXAMINATION OF AEDES ALBOPICTUS CLONE C6/36 CELLS INFECTED WITH DENGUE VIRUS 2 AT ELEVATED INCUBATION TEMPERATURE

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Summary. – Morphological changes such as loss of cytoplasm, membrane destruction and vacuolar swelling in *Aedes albopictus* clone C6/36 cells infected with dengue virus 2 (DV-2) New Guinea B strain and incubated at 28°C and 37°C, were observed under electron microscope. Both infected and uninfected cells showed significant damage at 37°C in contrast to normal appearance at 28°C. A higher number of virus particles were observed in the cytoplasm at 37°C than at 28°C.

Key words: dengue-2 virus; mosquito cells; elevated temperature; cytopathology

Introduction

Dengue virus (DV) infection has become a serious public health problem in the tropics because of increasing number of patients, expanding epidemic areas and appearance of severe clinical manifestation like dengue haemorrhagic fever (DHF)/dengue shock syndrome (World Health Organization, 1966; Halstead, 1966, 1980, 1992, 1993). The laboratory diagnosis of DV infection is important for accurate information of clinicians, epidemiologists and health administrators for proper case management and control of the illness (Igarashi, 1994). Classical haemagglutination-inhibition (HI) test (Clarke and Casals, 1958; Shope and Sather, 1997; World Health Organization, 1986) which has been a gold standard in the dengue serology has gradually been replaced by or supplemented with an enzyme-linked immunosorbent assay of IgM (IgM-ELISA) (Burke, 1983; Bundo and Igarashi, 1985; Lam *et al.*, 1987; Innis *et al.*, 1989).

In the HI test, antigens extracted from infected suckling mouse brains were used (Clarke and Casals, 1958) which is laborious, time consuming, and requiring large volumes of organic solvents and handling of animals.

The effect of elevated incubation temperature on the DV antigen production in infected *Ae. albopictus* C6/36 cell cultures has been investigated in our laboratory in the search for a better condition for the antigen production. Previous studies showed that an increased incubation temperature led to an increased DV-2 and DV-3 antigen production in the extracellular medium as detected by ELISA (Mohamed *et al.*, 1995) as well as an accumulation of extracellular and intracellular DV RNA (Mangada *et al.*, 1995).

In this study, the ultrastructural changes in *Ae. albopictus* C6/36 cells infected with DV-2 virus New Guinea B strain and incubated at two different temperatures have been investigated.

Materials and Methods

Cells. Ae. albopictus C6/36 cell line (Igarashi, 1978) was grown in tissue culture flasks at 28°C in Eagles's Medium containing Earle's salts supplemented with 10% heat-inactivated foetal serum (FCS) and 0.2 mmol/l each of non-essential amino acids.

Abbreviations: DHF = dengue haemorhagic fever; DV = dengue virus; FCS = foetal calf serum; HI = haemagglutination-inhibition; HRP = horseradish peroxidase; IgM-ELISA = enzyme-linked immunosorbent assay of IgM; FFU = focus forming unit; PBS = phosphate-buffered saline; p.i. = post infection

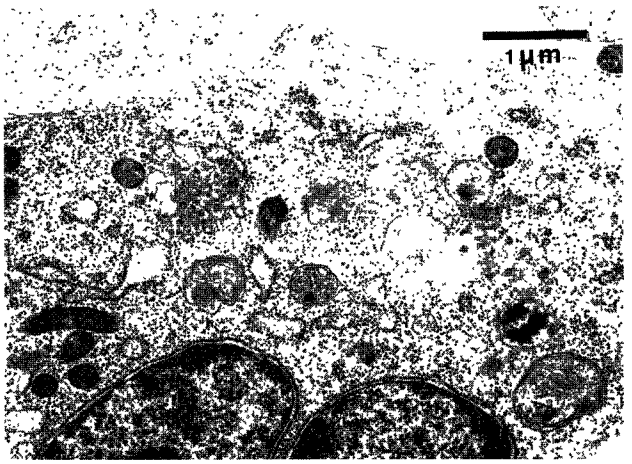

Fig. 1
Mock-infected C6/36 cells on day 4 at 28°C
Intact cellular structure.

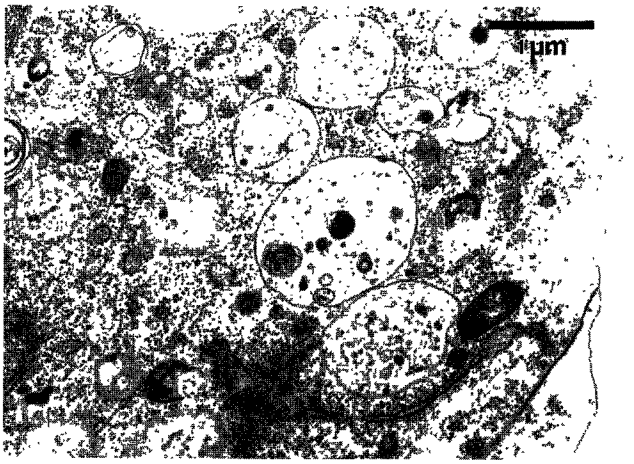

Fig. 2
DV-2-infected C6/36 cells on day 4 at 28°C
Intact cellular structure.

Virus. DV-2 New Guinea B strain that underwent multiple passages in *Ae. albopictus* clone C6/36 cells was used in this study. The stock virus was aliquoted and stored at -80°C until use.

Virus infectivity titration by focus formation. The microfocus method of Okuno *et al.* (1985) with certain modifications was used. Approximately 10^4 of BHK-21 cells were inoculated per well into 96-well microplates (Nunc), and incubated at 37°C in 5% CO_2 atmosphere. Serial ten-fold dilutions of virus specimens were prepared in the growth medium containing 5% of FCS. When the cell sheet became confluent, the growth medium was removed and the virus specimen dilutions (25 µl/well) were inoculated in the wells in triplicate. The virus adsorption proceeded for 2 hrs at 37°C with agitation every 30 mins. The cultures were then overlayed with 100 µl of the overlay medium (0.5% methyl cellulose 4,000 in the maintance medium (Eagle's Medium with Earle's salts and 2% FCS)). The plates were then incubated at 37°C for 70 hrs. Infected cells were visualized by immunoperoxidase staining. Briefly, after the overlay medium was removed, the plates were rinsed with phosphate-buffered saline (PBS) and the cells were fixed with 5% formaldehyde in PBS for 20 mins. After rinsing, the cells were permeabilised with 1% Nonidet P-40 in PBS, incubated at room temperature for 20 mins, and blocked with Block Ace (Ukijirushi-Nuygyo, Japan). After rinsing, the plates were successively reacted with DHF patient's serum at 1:500 dilution and horseradish-peroxidase (HRP)-conjugated anti-human IgG (Cappel, USA) at 1:500 dilution, for 1 hr each. The HRP reaction proceeded for 5 mins with a substrate solution containing 0.5 mg/ml 3,3'-diaminobenzidine tetrahydrochloride and 0.02% H_2O_2. The plates were rinsed with tap water and brown foci (virus-infected cells) were counted using an inverted microscope (CK Olympus, Japan). Infectivity titers were expressed in focus forming units (FFU) per ml.

Experimental cell infection. Ae. albopictus clone C6/36 cell line was grown at 28°C in Eagle's Medium with Earle's salts in 24-well-microplates (Falcon, USA) covered with a sheet of celophane. Approximately 1.5 x 10^5 cells were inoculated in 1 ml per well. When the cell sheet became confluent, the growth medium was removed and 400 FFU of the virus in 100 µl per well was inoculated in plates in duplicate. Mock-infected plates served as controls. The virus adsorption proceeded for 2 hrs at 28°C with agitation every 30 mins. Thereafter the cells were covered with 1 ml of the maintenance medium per well and incubated at 28°C and 37°C.

Sample preparation. On days 4 and 7 post infection (p.i.), the supernatants of the infected as well as of the mock-infected cells were collected and stored at 4°C for DV antigen assay. The cellophane sheets carrying the C6/36 cells were processed for morphological studies and observed under an electron microscope.

Electron microscopic examination. The cellophane sheets carrying the cells were treated with a solution of 2% glutaraldehyde and 0.01% $CaCl_2$ in 0.01 mol/l sodium cacodylate pH 7.2. Three washings of the sheets were done with the cacodylate buffer alone by centrifuging at 450 x g for 15 mins each time. A postfixation was done in 1% OsO_4 in the cacodylate buffer for 20 mins at room temperature.

The fixed specimens were dehydrated through graded ethanol solutions and finally in absolute acetone. Then they were infiltrated and embedded in epoxy resin. Ultrathin sections were obtained using a Reichert Ultra Cut E ultramicrotome and stained with uranyl acetate and lead citrate. The specimens were observed and photographed under a JEM 100 CX electron microscope (JEOL Ltd., Tokyo) at an acceleration voltage of 80 kV.

Results and Discussion

The electron microscopic examination of C6/36 cells infected with DV-2 at 28°C and then incubated at 28°C and 37°C in comparison with mock-infected controls yielded the following results.

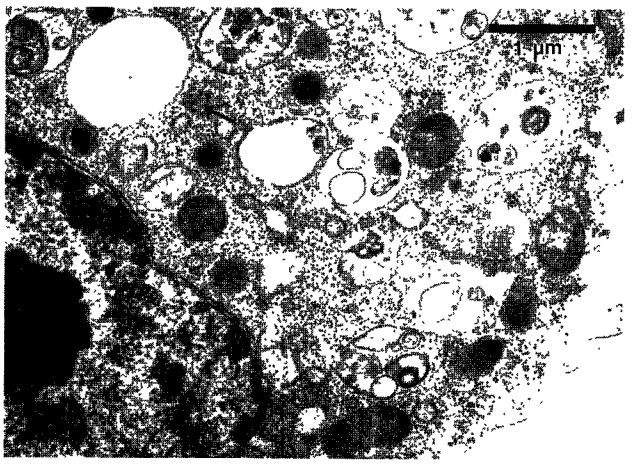

Fig. 3
Mock-infected C6/36 cells on day 7 at 28°C
Intact cellular structure except empty vacuoles (arrows)

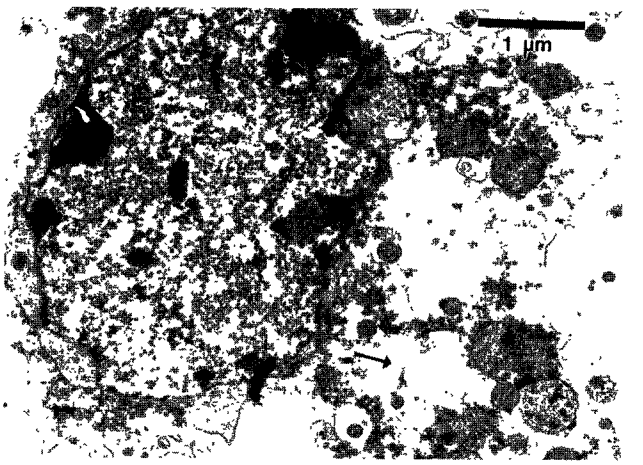

Fig. 5
Mock-infected C6/36 cells on day 4 at 37°C
Damaged cellular structure, empty vacuoles (arrows).

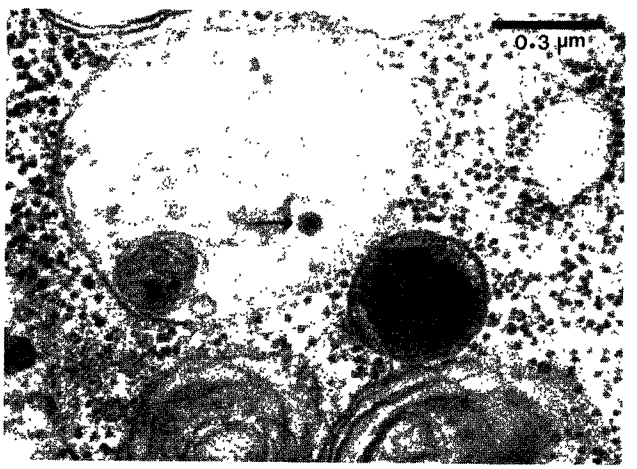

Fig. 4
DV-2-infected C6/36 cells on day 7 at 28°C
Intact cellular structure except empty vacuoles. A few virions in the cytoplasm (arrows).

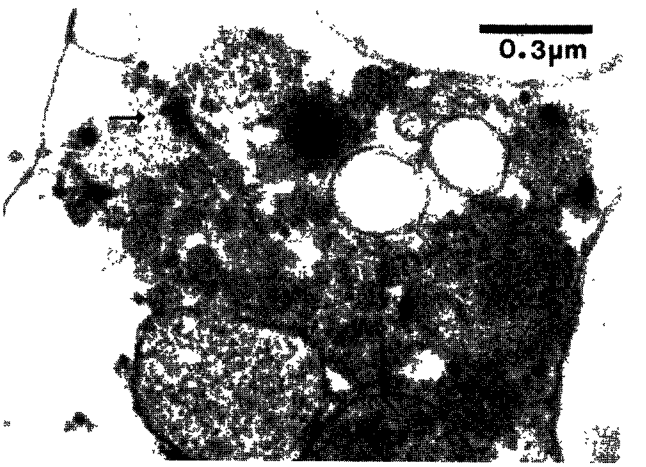

Fig. 6
DV-2-infected C6/36 cells on day 4 at 37°C
Destructed cellular structure, a few virions in the cytoplasm (arrows).

At 28°C, the morphology of both the virus-infected and non-infected cells remained unchanged throughout the observation period of 7 days (Figs. 1, 2, 3 and 4). Namely, the cells displayed intact nuclear and organelle membranes, numerous free ribosomes attached to the endoplasmic reticulum but empty vacuoles in the cytoplasm. A few free viral particles of 40 – 55 nm in diameter were seen in the cytoplasm of infected cells on day 4 while more of them on day 7.

At 37°C, significant changes were noticed already on day 4 in both the virus-infected and non-infected cells (Figs. 5 and 6). Namely, disappearance of nuclear membrane and loss of cytoplasmic material in general and of ribosomes in particular were seen. More virus particles were observed as compared to 28°C. Later, on day 7, both the virus-infected and non-infected cells underwent lysis, loss of structural integrity (Figs. 7 and 8). The number of free or clustered viral particles in the cytoplasm in the infected cells increased and was higher as compared to 28°C (Fig. 8).

A study on the influence of higher temperature on DV-2-infected C6/36 cells carried out by Corner *et al.* (1987) showed that the elevated temperature accelerated the onset of cytopathic effect and the formation of virus-specific proteins, and also enhanced the titre of extracellular infectious virus. A similar study carried out by Mohamed *et al.* (1995) using a micro-

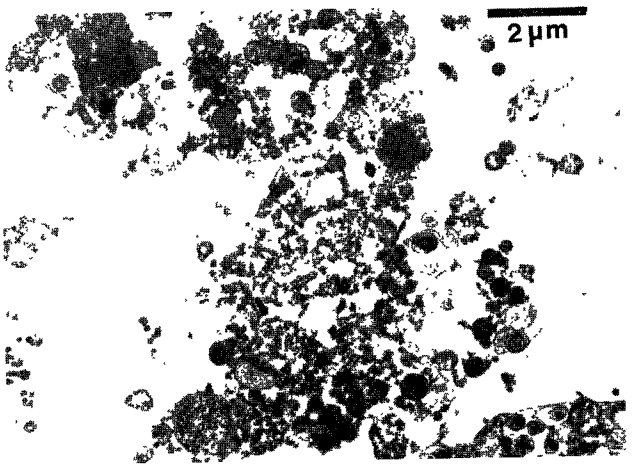

Fig. 7
Mock-infected C6/36 cells on day 7 at 37°C
Loss of cellular integrity.

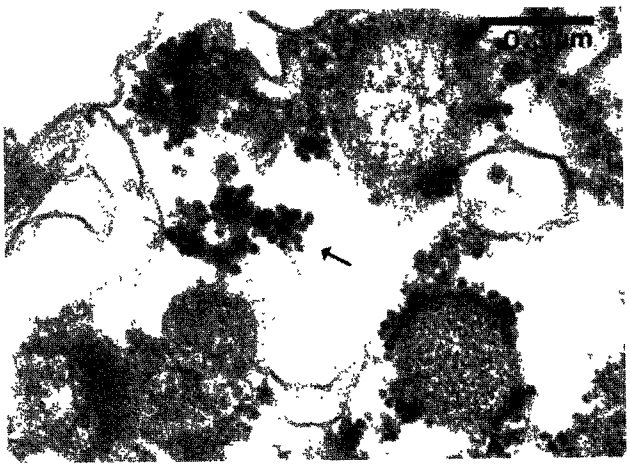

Fig. 8
DV-2-infected C6/36 cells on day 7 at 37°C
Destructed cellular structure, numerous virions in the cytoplasm (arrows).

sandwich method in the search for a better condition of virus propagation showed that the elevated incubation temperature enhanced the antigen production of prototype DV-2 and DV-3. Another study carried out by Mangada *et al.* (1995) using quantitative reverse transcription/polymerase chain reaction showed that incubation of DV-2-infected C6/36 cells at an elevated temperature led to an increased viral RNA synthesis.

In the present study, we observed the various ultrastructural changes in C6/36 cells infected with DV-2 at the elevated temperature. The results showed that the increased incubation temperature led to the significant destruction of cell structure and higher number of virions in contrast to the lower number of virions and healthy appearance of cells at 28°C. The incubation of C6/36 cells at 28°C has been a common practice as this temperature is apparently optimal for the cell growth (Mangada *et al.*, 1995). An increase of temperature up to 37°C may lead to the destruction of cell architecture. A higher titer of extracellular DV-2 at 37°C observed by Corner *et al.* (1987) was attributed to this temperature which triggered off the cellular homeostatic mechanisms in C6/36 cells and made the cells more efficient in supporting virus replication. A similar phenomenon was observed also in our present experiments.

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