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Electron Microscopic Observations on *Aedes albopictus* Cells Infected With Dengue Viruses

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With 6 Figures

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Summary

In dengue virus infected *Aedes albopictus* cells, electron-dense particles, larger than single ribosomes, were arranged on the cytoplasmic sides of rough endoplasmic reticulum (RER) membranes. Mature virions 40—45 nm in diameter as well as vesiculotubular structures 50—120 nm in diameter appeared in enlarged cisternae of RER filled with fine granular substance. Many of the mature virions and somewhat degenerated vesiculotubular structures remained to be enclosed in membranous structures presumably derived from RER, even after degeneration of infected cells. The findings suggest that development of dengue viruses in cultured *A. albopictus* cells takes place in close relationship with the activated membranes of RER.

Other morphological changes observed in dengue infected *A. albopictus* cells were 1. electron-dense "double-track structures" in areas of virion morphogenesis, 2. fine crystalline structures in type-2 dengue infected cells, and 3. aggregates of nucleoid structures, in cells persistently infected with type 2 dengue virus. The implication and nature of these structures in relation to virion morphogenesis remain to be investigated.

Introduction

Dengue hemorrhagic fever has been one of the most important virus diseases in Southeast Asia and is caused by four different serotypes of dengue (DEN) virus (16, 17). The viruses belong to group B arboviruses, flavivirus of Togaviridae (27) and are transmitted by *Stegomyia* mosquitoes such as *Aedes aegypti* or *A. albopictus* between susceptible vertebrate hosts, mainly human beings (9).

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Studies on the fundamental process of the growth of virus in mosquito vectors as well as in vertebrate hosts are important in order to understand the basic biology and ecology of the arboviruses in nature. Morphological studies on the development of dengue viruses have been performed using electron microscopy in cultured vertebrate cells such as LLC-MK_2 (8, 12, 30), VERO (25), BHK21 (22, 45), human lymphoblastoid (46) or leukemic (24) cell lines as well as suckling mouse brains (32, 43, 44). However, such a study has not been reported in cultured mosquito cells.

In this report we describe the morphological changes observed in the virus-sensitive *A. albopictus* cell clone (19) acutely or persistently infected with dengue viruses.

Materials and Methods

Viruses

Following dengue viruses were used: type 1 (DEN-1) Hawaiian strain, type 2 (DEN-2) New Guinea B strain, type 3 (DEN-3) H-87 strain, and type 4 (DEN-4) H-241 strain. The origins and passage histories of the viruses were described elsewhere (19). Ten per cent homogenates of infected suckling mouse brains were prepared in Eagle's (13) minimal essential medium (MEM) containing 0.2 per cent of bovine plasma albumin fraction V (Armour, Ill. U.S.A.). The supernatants of centrifugation (10,000 rpm, 30 minutes) were stored at $-70°$ C as seed viruses. Titers of infective viruses were assayed by focus counting using the indirect fluorescent antibody method as described (19, 20). Rabbit antisera against DEN viruses were kindly supplied by Y. Okuno of this Department and were used at 1:150 dilution. Fluorescent isothiocyanate conjugated antirabbit 7S gammaglobulin goat globulin was obtained from Hyland Division Traverol Laboratories, Inc. Calif. U.S.A. and was used at 1:70 dilution. Titers were expressed as focus forming units (FFU) per ml.

Cells

The isolation of virus-sensitive clone C6/36 from Singh's *A. albopictus* cells (40) has been described (19). The cloned cells were grown at 28° C with 10 per cent fetal calf serum in E medium (MEM supplemented with 0.2 mM each of nonessential amino acids). BHK21 cells were grown at 37° C with 10 per cent calf serum in MEM.

Virus Infection

Replicate cultures of *A. albopictus*, clone C6/36, cells were prepared in 2 ounce rubber-stoppered bottles by seeding 5×10^5 cells in 5 ml of cell growth medium. After 3 days' incubation at 28° C, each bottle contained $3—5 \times 10^6$ cells forming monolayers. Medium was removed from each bottle and seed virus was inoculated (0.2 ml/bottle) at input multiplicities of 0.1 FFU/cell for DEN-1, DEN-3, and DEN-4 viruses, or 1 FFU/cell for DEN-2 virus. After 2 hours virus adsorption at 28° C, residual virus was removed and cell sheets were washed twice with 4 ml of phosphate buffered saline. Cells were incubated with 5 ml/bottle of virus maintenance medium (2 per cent fetal calf serum in E medium) at 28° C. Specimens were harvested every day up to 7 days after infection and infective virus released into the medium was assayed as described above.

Maintenance of Persistent Infection

On the 7th day after infection, infected cells were resuspended in cell growth medium at 1×10^5 cells/ml and were transferred into fresh 2 ounce bottles (5 ml/bottle). Incubation at 28° C was continued changing media once a week. When cells were grown up to fill the bottle, they were transferred once a week with 1:20 split.

Preparation of the Specimen for Electron Microscopy

Infected cell sheets in glass bottles, harvested at various times after the initial infection, were washed twice with 0.1 M phosphate buffer, pH 7.4 (PB). After prefixed

with 2 per cent glutaraldehyde in PB (4° C, 1 hour), cells were washed 3 times with 0.2 M sucrose in PB and were scraped into the same solution. Cells were collected by centrifugation (600 rpm, 5 minutes) and were postfixed with 1 per cent OsO_4 in Millonig's buffer (28) at 4° C for 1 hour. Fixed cells were pelletted by centrifugation (600 rpm, 3 minutes) and were rinsed briefly with distilled water and were stained with 2 per cent uranyl acetate in ethanol for 20 minutes. Specimens were dehydrated by graded ethanol series (50, 70, and 95 per cent, 10 minutes each) and were immersed in 1:1 mixture of ethanol and glycidyl-n-butylether (GBE) for 15 minutes, and then 1:1 mixture of GBE and epoxyresin for 30 minutes, respectively. Specimens were collected by centrifugation (1000 rpm, 5 minutes) and were transferred into BEEM capsules containing 100 per cent Spurr's epoxyresin (42). After 2 hours infiltration of the resin, specimens were sedimented to the bottom (2000 rpm, 5 minutes). Fresh resin was added and was polymerized at 60° C for 15 hours. Ultrathin sections of 50 to 90 nm thickness were prepared by LKB type 4801 ultramicrotome using glass knives. Sections were put on 200 mesh grid and were stained with uranyl acetate and bismuth nitrate (37), and were observed under Hitachi model HU-11DS electron microscope.

Results

Growth of Dengue Viruses in A. albopictus *Cells*

Figure 1 represents growth curves of 4 types of DEN viruses in cultured *A. albopictus*, clone C6/36, cells. After 1 day's lag period, infectious virus titer in the medium increased and reached plateau 4—5 days after infection. Maximum titers were around 10^6 FFU/ml for DEN-1, DEN-3, and DEN-4, and around 10^8 FFU/ml for DEN-2. In this experiment seed viruses were prepared from infected suckling mouse brains, however, the patterns of growth curves were similar to those obtained with seed viruses prepared from infected *A. albopictus* cells (19) except that the lag periods were somewhat longer.

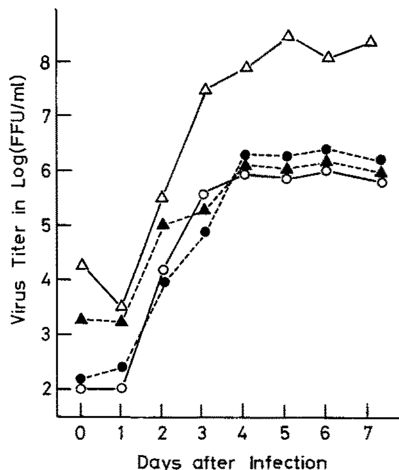


Fig. 1. Growth of dengue viruses in *A. albopictus* cells. Replicate cultures of *A. albopictus*, clone C6/36, cells prepared in 2 ounce bottles were inoculated with DEN-1 (○———○), DEN-2 (△———△), DEN-3 (● — — — ●), or DEN-4 (▲ — — — ▲) virus, and were incubated under virus maintenance medium at 28° C. Portions of infected culture fluids were harvested every day and assayed for infectious virus in order to provide virus growth curves as described in Materials and Methods

Morphological Changes in Acutely Infected A. albopictus *Cells*

Many rough endoplasmic reticulums (RER) were observed in uninfected *A. albopictus* cells, however, no virus-like particles were present inside the cytoplasm or in the cisternae of RER. Morphological changes observed in *A. albopictus*

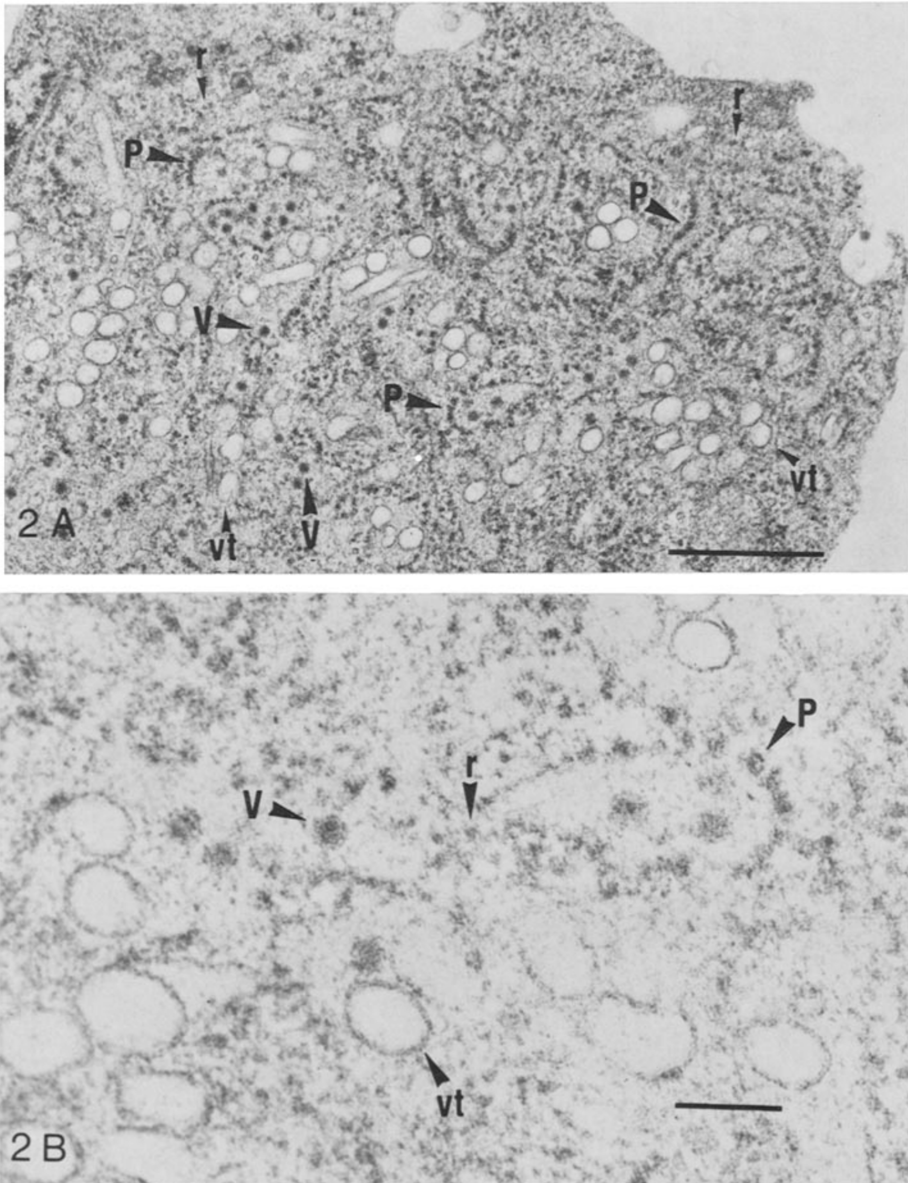


Fig. 2. *A. albopictus* cells 2 days after infection with DEN-1 virus. Arrangement of electron-dense particles *(P)*, larger than single ribosomes *(r)*, on the membranes of distended RER, which contain mature virions *(V)* and vesicular or tubular structures *(vt)*. Scale 500 nm in A and 100 nm in B

cells following DEN infections were essentially similar with every serotype of DEN viruses except for slight differences.

The earliest change observed one day after infection is the swelling of RER, and the arrangement of some electron-dense particles, about 2—3 times the size of single ribosomes, on the cytoplasmic sides of the RER membranes. The cisternae of RER were filled with fine granular substance, in which a few mature virions 40—45 nm in diameter and vesicular structures appeared. As the virus growth entered into logarithmic phase, the cisternae of RER increased in size, and the RER, electron-dense particles on their membranes, mature virions, and vesicular or tubular structures increased in number (Fig. 2A, B). These vesicular and tubular structures of 50—120 nm in cross sections are probably the same kind of structures with different planes of sectioning and are possibly derived from, by distension of their lumens, from those tubular structures of 50 nm diameter which contained low-electron-dense material along their membranes.

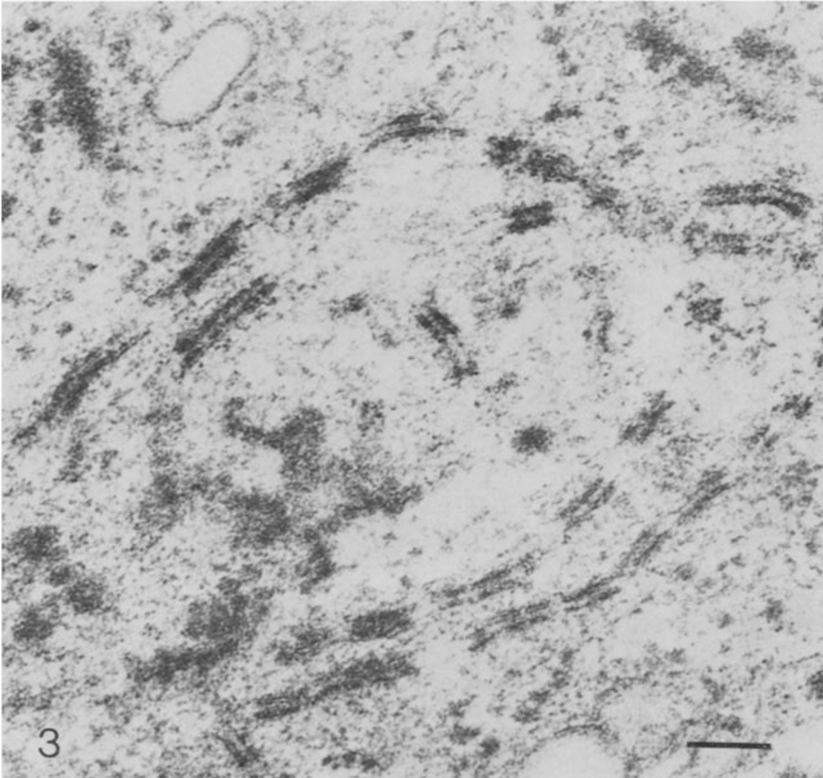


Fig. 3. "Double-track structures" arranged in circular form in *A. albopictus* cells 5 days after DEN-1 infection. Scale 100 nm

Sometimes, electron-dense parallel structures somewhat resembling desmosomes were observed in the area of virion morphogenesis. The structures are henceforce called "double-track" structures. Figure 3 shows highly developed "double-track"

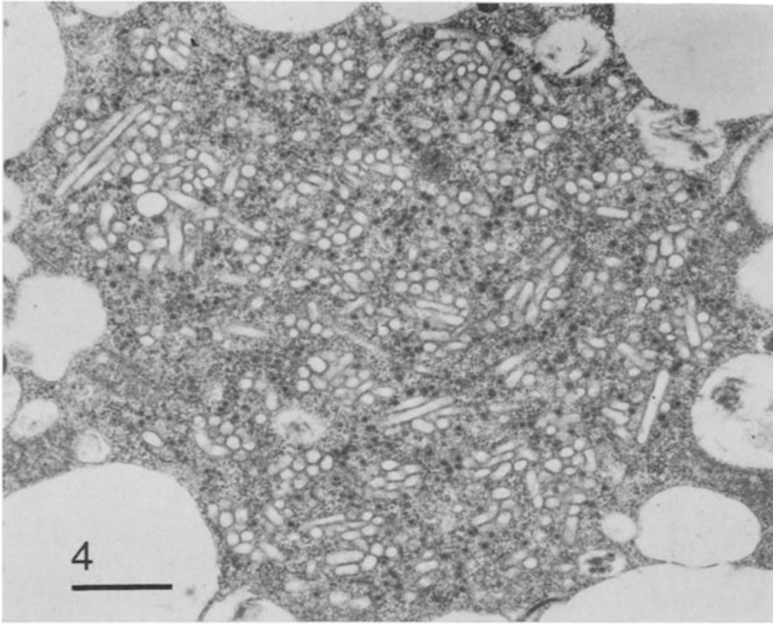


Fig. 4. Large numbers of vesiculotubular structures in *A. albopictus* cells 1 week after DEN-3 infection. Scale 500 nm

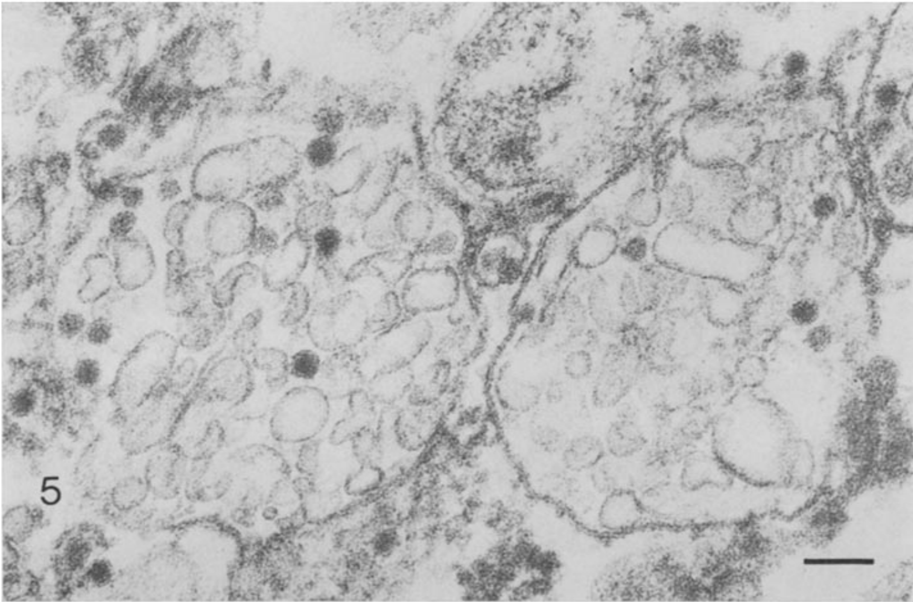
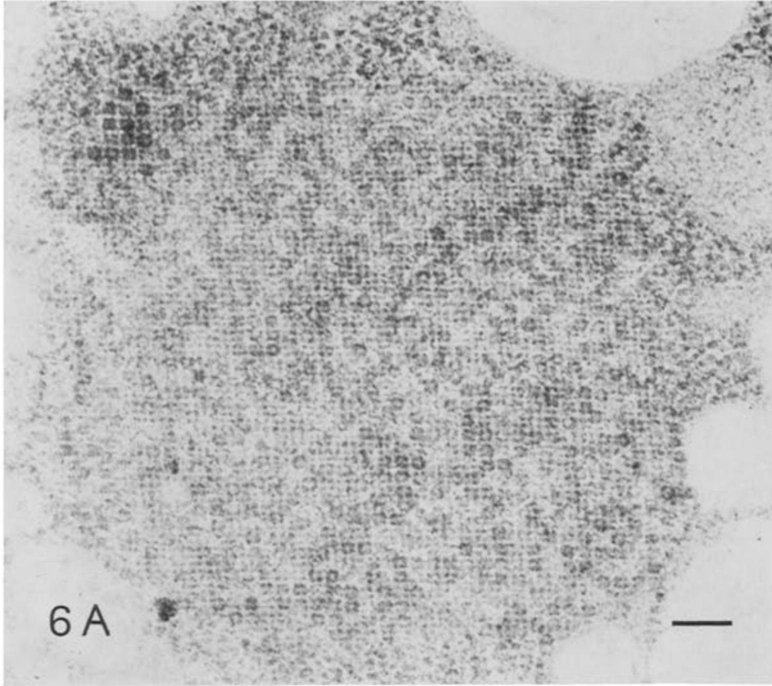


Fig. 5. Degeneration of *A. albopictus* cells and release of membranous structures containing mature virions and degenerated vesiculotubular structures. Two weeks after DEN-4 infection. Scale 100 nm



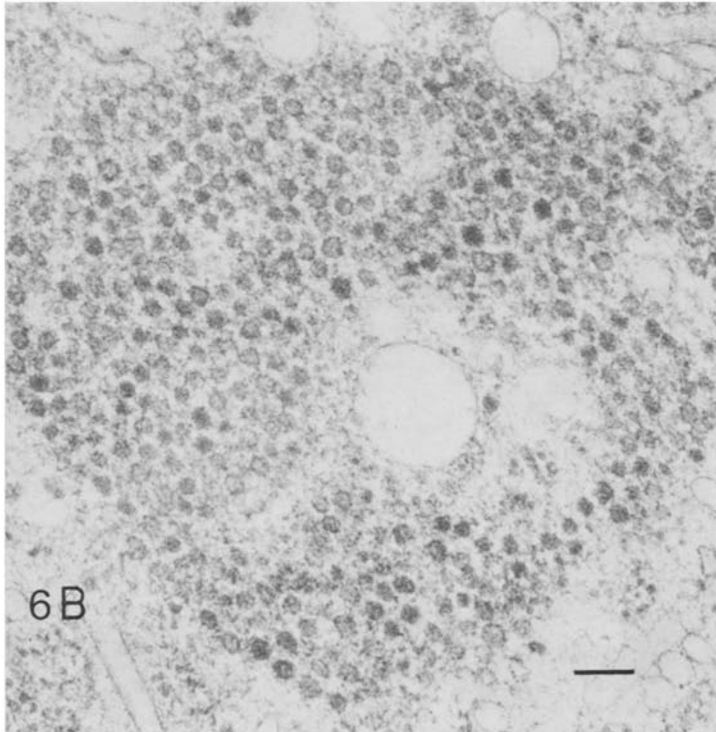


Fig. 6. *A. albopictus* cells infected with DEN-2 virus. *A* Crystal body in the cytoplasm with main spacing of 10 nm, observed 2 weeks after infection. *B* Crystalloid aggregates of 30 nm particles observed 10 weeks after infection. Scale 100 nm

structures arranged in circular form, which was observed 5 days after DEN-1 infection. A few mature virions were observed in close proximity to these structures. These structures were not observed in DEN-2, very rarely in DEN-3, and only occasionally in DEN-4 infected cells. Figure 4 shows large numbers of vesiculotubular structures found one week after DEN-3 infection.

After 1 week of infection, most of the cells showed some degree of degeneration, resulting in the release of some mature particles. However, many mature virions and vesiculotubular structures with somewhat degenerated appearance were still enclosed in smooth-surfaced membranous structures possibly derived from RER (Fig. 5).

Morphological Changes in Persistently Infected A. albopictus *Cells*

Observations were performed 2 weeks after infection and thereafter every 4 weeks up to 30 weeks after the initial infections. In the case of DEN-1, DEN-3, or DEN-4 infected cells, fundamental changes observed in persistently infected cells were similar to those found in acute infections, while in DEN-2 infected cells, fine crystalline structures were often observed 2 weeks after infection. The main spacing of the crystal was around 10 nm (Fig. 6A). Ten weeks after DEN-2 infection, crystalloid aggregates of particles 25—30 nm in diameter were observed in the area with many vesiculotubular structures and some "double-track structures" which were characteristic of DEN infected cells (Fig. 6B).

Discussion

Several reports have described dengue virus morphogenesis in cultured vertebrate cells (8, 12, 22, 24, 25, 30, 45, 46) and in vertebrate hosts (32, 43, 44). Most of these observations are consistent in the points that dengue virion morphogenesis takes place in close relationship with the intracellular membranes, especially RER membranes, and that mature virions are observed mostly in the cisternae of these membranous structures. Such fundamental characteristics of virion morphogenesis appear to be common features in flavivirus infected vertrebrate cells (1, 2, 3, 4, 5, 6, 7, 10, 11, 18, 23, 26, 29, 31, 34, 35, 36, 39, 41, 47, 49) or mosquito cells (14, 21, 48). Our observations in dengue infected *A. albopictus* cells are also consistent with these points. Schlesinger recently referred to the virions found in intracellular vacuolar membranous structures mentioning that these particles could be the dead end products of incompletely processed virions; he suggested that other possibilities including surface budding from plasma membrane should be considered for the dengue virion morphogenesis (38). Matsumura *et al.* observed budding of dengue virions from plasma membranes of infected human leukemic cell line (24). Also Oyama *et al.* observed similar surface budding of Japanese encephalitis virus from infected VERO cells (33). Active budding from plasma membranes was supposed to be related with high virus yields from these infected cells, however, we seldom observed pictures suggesting budding of precursor particles from plasma membranes of dengue infected *A. albopictus* cells in spite of high virus yields.

Although we observed electron-dense particles, larger than the size of single ribosomes, on the cytoplasmic side of RER membranes, we could not obtain de-

finite proof showing that these particles bud through RER membranes to become mature virions. These electron-dense particles resembled precursor particles observed in DEN-2 infected LLC-MK_2 cells (45) or those particles in "rosary body" in DEN-4 infected BHK21 cells (22), however, the possibility still remains that these particles could be aggregates of ribosomes.

In dengue infected *A. albopictus* cells, swelling of RER was observed, and their cisternae were filled with fine granular substance, in which mature virions as well as a large number of vesiculotubular structures appeared. Such remarkable vesiculotubular structures were not observed in dengue infected BHK21 cells, but were reported in *Culex pipiens* salivary glands infected with St. Louis encephalitis virus (48). Vesicular structures of similar appearance but fewer in number were also observed in distended cisternae of RER in dengue infected mouse brains (43, 44) or LLC-MK_2 cells (8, 12, 45). Also similar structures have been described in other flavivirus-infected cells or tissues (4, 5, 6, 7, 10, 29, 34, 36, 39, 49). The origin and function of these vesiculotubular structures are still unknown, although some authors suppose that they might be related to viral RNA synthesis (39) as in the case of alphaviruses (15). Others think that these structures could be incomplete capsid protein (6). Otherwise, they may be some nonspecific reaction of host cells to the infection with flaviviruses.

The mechanism of virus release from the cisternae of RER into the culture medium is also unknown. In some cases, however, some swollen RER containing mature virions lie closely beneath the plasma membrane. If membrane fusion takes place between the membrane of the RER and the plasma membrane, as a consequence, mature virions will be released into the medium, as sometimes suggested for the release of flaviviruses (10, 12, 25). Another possibility will be the disintegration of infected cells and disruption of the membranes enclosing mature virions and vesiculotubular structures. However, the limiting membrane of such structures appear to be fairly tight and remain after degeneration of infected cells. It might be necessary to disrupt those membranes to obtain maximum infectious titer of the viruses as suggested for St. Louis encephalitis virus (29).

Some other morphological changes in dengue-infected *A. albopictus* cells are 1. double-track structures existing in close proximity to the area of virion morphogenesis, 2. fine crystals with main spacing of 10 nm, and 3. crystalloid aggregates of 25—30 nm particles. The first structure was especially remarkable in DEN-1 infected cells and the latter two structures were observed in DEN-2 infected cells, though lattice crystals have been reported from DEN-3 infected LLC-MK_2 cells (30). The particle size of the aggregate corresponded to the core size of the virion, and crystalloid aggregates of incomplete particles was observed in DEN-2 infected Raji cells (46). However, the nature and function of these structures in relation to dengue virus morphogenesis still remain to be studied in further detail.

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