Electron Microscopic Observation of a Newly Isolated Flavivirus-like Virus from Field-caught Mosquitoes

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SUMMARY

Of many unidentified virus strains which were isolated from field-caught mosquitoes by using C6/36 cells (a virus-sensitive clone of Aedes albopictus cells), three strains which formed small size plaques (SP virus) in C6/36 cells were investigated by electron microscopy. Although the SP virus strains did not react with antisera against known arboviruses in serological tests, they closely resembled flaviviruses in morphology. However, when they were compared to Japanese encephalitis (JE) virus, several differences in morphogenesis were observed. Proliferating membranous structures and electron-dense amorphous areas involving precursors of the virus were observed only in cells infected with the SP virus strains. Enlarged areas of endoplasmic reticulum containing mature virions were often observed adjacent to these structures. Since the SP virus strains were isolated from wild mosquitoes and multiplied only in mosquito cells, it seems appropriate to classify them as insect viruses which resemble togaviruses morphologically.

In 1978, we attempted virus isolation from female Culex tritaeniorhynchus mosquitoes using Aedes albopictus clone C6/36 cells (Igarashi et al., 1981). In addition to Japanese encephalitis (JE) and Getah viruses, many infectious agents which produced various sizes of plaques on C6/36 cells were isolated. Since viruses producing small size plaques (SP virus) were most frequently observed (69.1% of the pools tested) among the unidentified viruses, their characterization seemed to be important. For the present study, we chose three strains of SP virus (B-40, D-36 and I-8) which were isolated at different sites in the suburban areas around Osaka City. These strains were plaque-purified three times in C6/36 cells. For comparison, the E-50 strain of JE virus which had been isolated from female C. tritaeniorhynchus using C6/36 cells at Mihara City in Osaka Prefecture in 1979 was used.

A. albopictus clone C6/36 cells (Igarashi, 1978) were used for virus propagation, infectivity assay and electron microscopic study. Cells were cultured in Eagle's minimal essential medium (MEM) supplemented with 10% foetal calf serum and 0.2 mM each of non-essential amino acids (NEAA) (growth medium). Cells were kept at 28°C.

Stock viruses were prepared in C6/36 cells. After virus adsorption, cells were incubated in MEM supplemented with 2% foetal calf serum and 0.2 mM each of NEAA (maintenance medium). Six days after virus infection, culture medium was harvested and divided into aliquots for storage in liquid nitrogen.

For plaque titration, Petri dishes (6 cm diam.) containing a monolayer of C6/36 cells were infected with 0.2 ml of serial dilutions of the virus stocks. After 2 h of adsorption, cells were covered with the first overlay medium [MEM, 1% Noble Agar (Difco), 0.04% DEAE-dextran, 2% foetal calf serum, 0.2 mM each of NEAA] and were incubated in a CO2 incubator at 28°C for 5 days. After this incubation, cells were covered with the second overlay medium (0.012% neutral red in the first overlay medium) and plaques were counted on the following day. Fig. 1
Short communication

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Fig. 1. Plaques of SP virus strains (B-40, D-36 and I-8) and the E-50 strain of JE virus produced on A. albopictus C6/36 cells, 6 days after inoculation.

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Fig. 2. Growth curves of SP virus strains (B40, ●; D-36, ▲; I-8, ■) and the E-50 strain of JE virus (○). These viruses were inoculated onto C6/36 cells and titrated as described in the text.

shows plaques of three strains of SP virus and the E-50 strain of JE virus. The plaques of SP virus were clear and homogeneous in morphology, and 0.5 to 1.0 mm in diameter. The plaques of JE virus were 3 to 5 mm in diameter.

Growth characteristics of SP and JE viruses in C6/36 cells were investigated and the results are shown in Fig. 2. The growth curves of the three strains of SP virus were almost identical, each reaching its highest level of approx. $2 \times 10^7$ p.f.u./ml at 3 days after inoculation. The titre of JE virus strain E-50 was higher, reaching a peak of approx. $5 \times 10^8$ p.f.u./ml at 3 days after inoculation. The monolayer of C6/36 cells infected with JE virus strain E-50 showed marked syncytium formation by 3 days post-infection. The cells infected with SP virus became rounded by 6 days post-infection.

To investigate whether SP virus strains have the ability to infect mammalian hosts, the strains were inoculated into suckling mice intracerebrally, and into cultured cells of mammalian origin (BHK-21, Vero and RK-13). Although all the mice inoculated with the JE virus died 6 days after inoculation after showing typical paralytic symptoms from day 3 onwards, mice inoculated with SP virus strains did not show any sign of infection even 21 days after inoculation. When
mammalian cells were infected with the JE virus and culture fluids were titrated on C6/36 cells, titres were $4 \times 10^5$ p.f.u./ml for BHK-21, $2.4 \times 10^6$ p.f.u./ml for Vero and $6.0 \times 10^7$ p.f.u./ml for RK-13. However, no plaques were observed when the culture fluids from SP virus-infected cells were titrated.

To identify SP virus strains serologically, complement fixation (CF) and plaque reduction neutralization (N) tests were performed using antisera of 30 arbovirus grouping fluid supplied by the NIH, U.S.A. Antisera against bovine virus diarrhoea, hog cholera virus and equine arteritis virus were supplied by Dr Y. Inaba, National Institute of Animal Health, Japan. Antisera against the cell-fusing agent isolated from a cell line of *A. aegypti* (Stollar & Thomas, 1975) were supplied by Dr V. Stollar (Rutgers Medical School, N.J., U.S.A.). The SP virus strains did not react at all with any of these antisera in CF and N tests.

Procedures for electron microscopic observation were as follows. Monolayers of C6/36 cells were infected with the SP virus or the JE virus at an input multiplicity of 0-1 p.f.u./cell. Mock-infected cells were also prepared. After 2 h of adsorption, the infected cells were cultured in maintenance medium for 6 days at 28 °C. Thereafter, the infected cells were washed once with 0-1 M-phosphate buffer (pH 7-4), scraped off and centrifuged (2000 rev/min, 5 min) to form a pellet. The pellets were fixed for 1 h in 2% glutaraldehyde, washed and post-fixed for 1 h in 1% osmium tetroxide. They were then dehydrated in a graded ethanol series and propylene oxide, and embedded in epoxy resin. Ultrathin sections were cut with a diamond knife on an LKB ultramicrotome and stained with both uranyl acetate and lead citrate. For negative staining, the viruses were purified following the method of Igarashi *et al.* (1973) where polyethylene glycol 6000 was used for virus concentration, and ultracentrifugation in sucrose of 40% and 5% was carried out for virus purification. After the viruses were taken from the interphase of the two

Fig. 3. Thin section of a C6/36 cell infected with SP virus. Complete virus particles are observed in distended ERs and a perinuclear space (V). Proliferating membranous structures (M) were observed adjacent to the distended ERs. Bar marker represents 1 μm.
Fig. 4. Comparison of SP and JE virus morphology. In thin sections, both SP (a) and JE (b) virus particles are composed of an outer membrane and an electron-dense nucleoid. In negative staining, SP virus (c, d) and JE virus (e, f) were stained by PTA (c, e) or uranyl acetate (d, f). Outer membrane structure is well preserved by PTA and core structure is well resolved by uranyl acetate. Bar markers represent 50 nm.

sucrose layers, they were stained with 2% phosphotungstic acid (PTA) or 2% uranyl acetate. The samples were observed in a Hitachi model HU-12 electron microscope.

The morphology and morphogenesis of all three strains of SP virus in C6/36 cells were similar. No virus-like particles were observed in mock-infected cells. Fig. 3 shows a whole cell infected with the SP virus; virus particles are present in distended endoplasmic reticulum (ER) and the perinuclear space (V) together with proliferating membranous structures (M).

Comparisons between the morphology of SP virus and that of JE virus are shown in Fig. 4. In thin sections, the shape and size of SP virus (a) and JE virus (b) are similar and virions 45 to 50 nm in diameter are composed of an outer membrane and an electron-dense nucleoid 30 nm in diameter. In negative staining, SP virus (c, d) and JE virus (e, f) were both stained by PTA (c, e) or uranyl acetate (d, f). The outer membrane structure of the virus particles is well preserved by PTA, showing projections on the surface. On the other hand, although the outer membrane is damaged, the core structure of the virus is well resolved by uranyl acetate.

As shown in Fig. 5, the most striking observation in cells infected with SP virus are electron-dense amorphous areas adjacent to distended ER where many virus particles are present. This area seems to be analogous to the membranous structure shown in Fig. 3. Since electron-dense particles (Fig. 5, arrows), which are presumed to be precursors of the virus, were observed in these areas, they may be sites of virus maturation and of release of mature virus particles into enlarged ERs. Some of the mature viruses might be transported to the perinuclear space, as it has connections with cisternae of the ER. Small particles on the margin of the distended ER seem to be crystallized ribosomes which are not related to virus cores, because their diameters were smaller (20 nm) than the nucleoid of mature virions (30 nm). None of the structures illustrated in Fig. 5 was observed in cells infected with the JE virus. Budding forms were not observed in cells infected with either SP or JE viruses.

In our preliminary experiments, SP virus was demonstrated to be composed of three main structural proteins by SDS–polyacrylamide gel electrophoresis, but with different electrophoretic mobilities from those of JE virus.
Fig. 5. Electron-dense amorphous areas where electron-dense particles (arrow) are involved. Many virions of the SP virus are present in the distended ER adjacent to the area. Bar marker represents 200 nm.

At present, we would propose classifying SP virus as an insect or mosquito virus belonging to the family Togaviridae. In order to clarify the nature of the virus further, physicochemical, biochemical and more serological investigation is needed.

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REFERENCES


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