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Electron Microscopy of Japanaut and Tilligerry Viruses: Two Proposed Members of the Orbivirus Group

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Abstract

Japanaut and Tilligerry viruses were studied by thin-section and negative-contrast electron microscopy, and from their morphology and morphogenesis both appear to be typical orbiviruses.

Characteristic intracellular structures are associated with the development of the two viruses. These structures include coated tubules and one or more types of structure which appear to be composed of a number of parallel sheets of moderately electron-dense material.

Introduction

Japanaut virus* was isolated from the blood of a bat (*Syconycteris crassa*) from the Sepik district of Papua New Guinea (I. D. Marshall, personal communication). Tilligerry virus, isolated from a pool of *Anopheles annulipes* mosquitoes from the Nelson Bay region of northern New South Wales, has been found to be serologically related to, but distinct from, the arbovirus Eubenangee (Gard *et al.* 1973).

This paper reports observations made by thin-section and negative-contrast electron microscopy on particle morphology and intracellular structures associated with the development of Japanaut and Tilligerry viruses in porcine stable–equine kidney cells.

Material and Methods

Viruses

The virus strains Japanaut (MK 6357) and Tilligerry (NB 7080) were kindly supplied by Dr I. D. Marshall, Department of Microbiology, John Curtin School of Medical Research, Canberra, as freeze-dried stock of suckling mouse brain. Japanaut virus in the sixth passage of the suckling mouse brain and Tilligerry virus in the fifth brain passage were used to infect cell cultures.

Cell Cultures

The porcine stable–equine kidney (PS–EK) cells were kindly supplied by Dr T. J. Bagust, Division of Animal Health, CSIRO, Parkville, Vic. The cell line almost certainly originated from a contamination of foetal EK diploid cells by PS cells (T. J. Bagust, personal communication). Monolayers of these cells were grown in medium 199 containing 5% foetal calf serum. After infection, the monolayers were maintained on the same medium but with 2% foetal calf serum.

Electron Microscopy

For thin-section electron microscopy, infected cells were harvested by scraping from the glass with a rubber policeman, then pelleted by low-speed centrifugation, and then fixed in Millonig buffered 4% (v/v) glutaraldehyde for 2 h at 4°C. After post-fixation in Millonig buffered 1%

*Japanaut virus is registered in the Catalogue of Arthropod-borne Viruses.

(w/v) osmium tetroxide for 1 h at 4°C the specimens were dehydrated in acetone and embedded in Araldite. Sections were cut on a Huxley ultramicrotome and stained with 1·0% (w/v) uranyl acetate and then with lead citrate 0·2% (w/v) in 0·1M sodium hydroxide.

For negative-contrast electron microscopy, virus-infected cells were similarly pelleted by low-speed centrifugation and a portion of the pellet was resuspended in a small volume of distilled water. Grids were briefly floated upside-down on the suspension, the excess liquid was blotted off and the grids were then stained with 1% (w/v) potassium phosphotungstate at pH 6·0 or 7·0. Alternatively a 20% (w/w) infected homogenate from the suckling mouse brain was examined after staining as outlined above.

Micrographs were taken using a Hitachi HU-11A electron microscope operating at 50 kV. Magnifications were calibrated using catalase crystals (Luftig 1967).

Results

Morphologically, Japanaut and Tilligerry viruses appeared to be very similar. In thin sections of infected PS–EK cells, virions characteristically appeared within and around discrete fibrillogranular intracytoplasmic inclusions (Figs 1–3). The virus particles themselves were approximately spherical and possessed an extremely electron-dense core surrounded by a more electron-lucent capsid layer (Figs 2, 4 and 5). The average diameter of Japanaut virus particles was 62 $\pm$ 4 nm (core 36 $\pm$ 3 nm) while that of Tilligerry virus particles was 63 $\pm$ 4 nm (core 36 $\pm$ 3 nm).

With both Japanaut and Tilligerry viruses, the release of virus from the infected cell seemed to occur mainly by cell dissolution which resulted in the release of unenveloped particles. Particles of each virus did occasionally gain envelopes by a budding process at the cell margin (Figs 4 and 5). For the particles with extracellular envelopes, the average diameters were 98 $\pm$ 4 nm for Japanaut virus and 100 $\pm$ 4 nm for Tilligerry virus.

As well as the fibrillogranular inclusions, tubular structures in the size range of spindle tubules (microtubules) and covered with a moderately electron-dense material were frequently seen in the cytoplasm of PS–EK cells infected with Japanaut virus (Fig. 1). These structures could possibly be interpreted as 'coated' tubules (Dales 1963). Although such tubules were rarely observed to be in direct contact with virus particles, they were never seen in uninoculated control cells.

In PS–EK cells infected with Tilligerry virus, structures possibly composed of two or more parallel sheets were occasionally observed within the intracytoplasmic fibrillogranular inclusions (Fig. 2).

Quite frequently structures rather different in appearance and apparently more extensive than those mentioned above were observed within the intracytoplasmic inclusions in PS–EK cells infected with Tilligerry virus (Fig. 3). These structures, which may also be composed of a number of parallel sheets, were also seen free in the cytoplasm, unassociated with the fibrillogranular inclusions.

From negative-contrast electron microscopy, both Japanaut and Tilligerry virus particles appeared to be composed of an inner capsid with an obvious capsomere structure which was often surrounded by an amorphous outer layer of rather variable thickness. This outer layer obscured the capsomeres and increased the diameter of the particles. The inner capsid exhibited large 'doughnut' or ring-like capsomeres such as are typical of the orbiviruses (Borden *et al.* 1971; Murphy *et al.* 1971). The actual capsomere number appeared small, possibly 32, although this cannot be claimed unequivocally at this stage. Since the particles were indistinguishable in general appearance from other orbiviruses, such as were illustrated by Lecatsas and

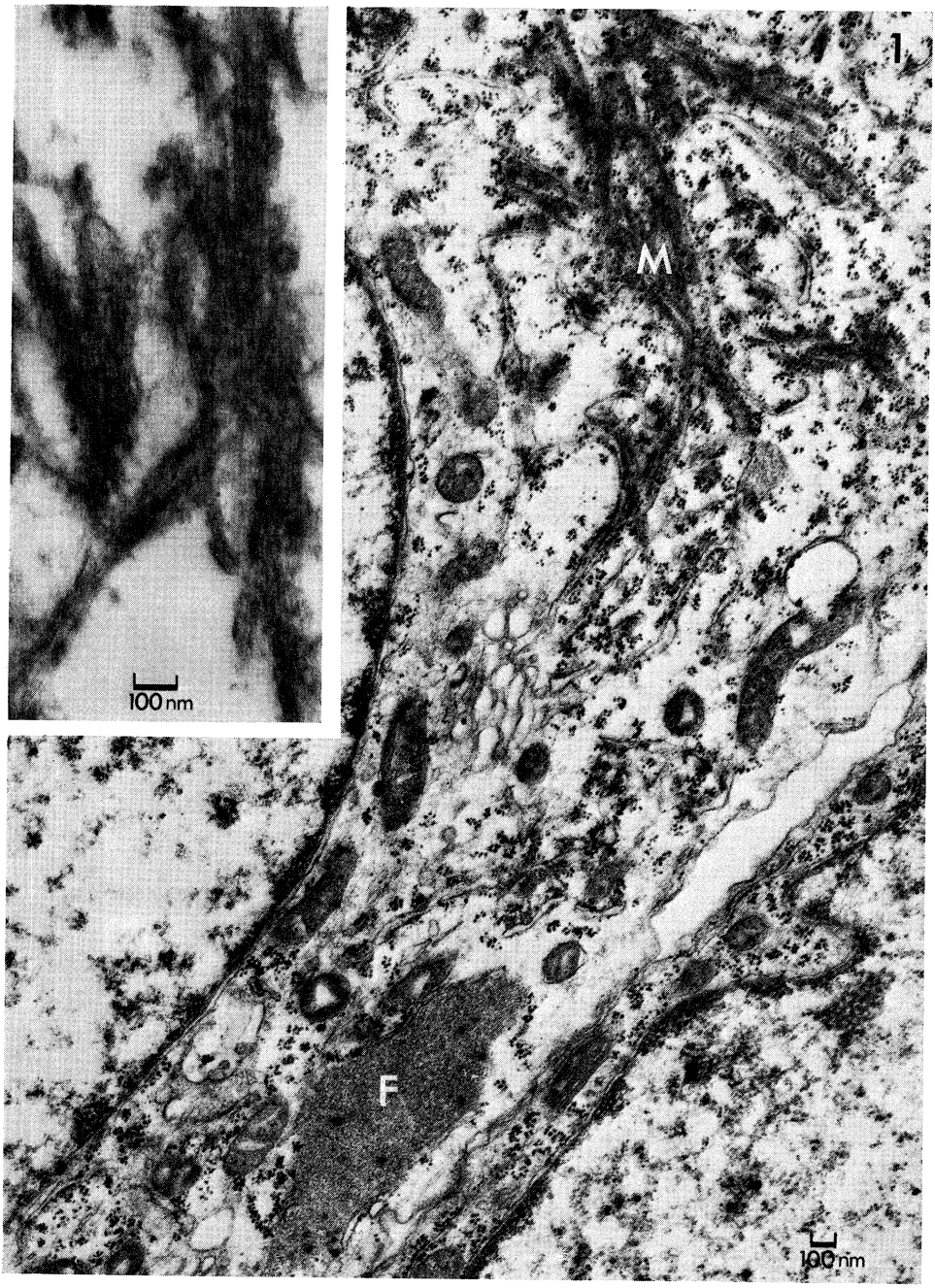


Fig. 1. Microtubule-like structures (*M*) covered with a moderately electron-dense material in the cytoplasm of a PS–EK cell infected with Japanaut virus. Note the fibrillogranular inclusion (*F*). Insert: Higher magnification of microtubule-like structures.

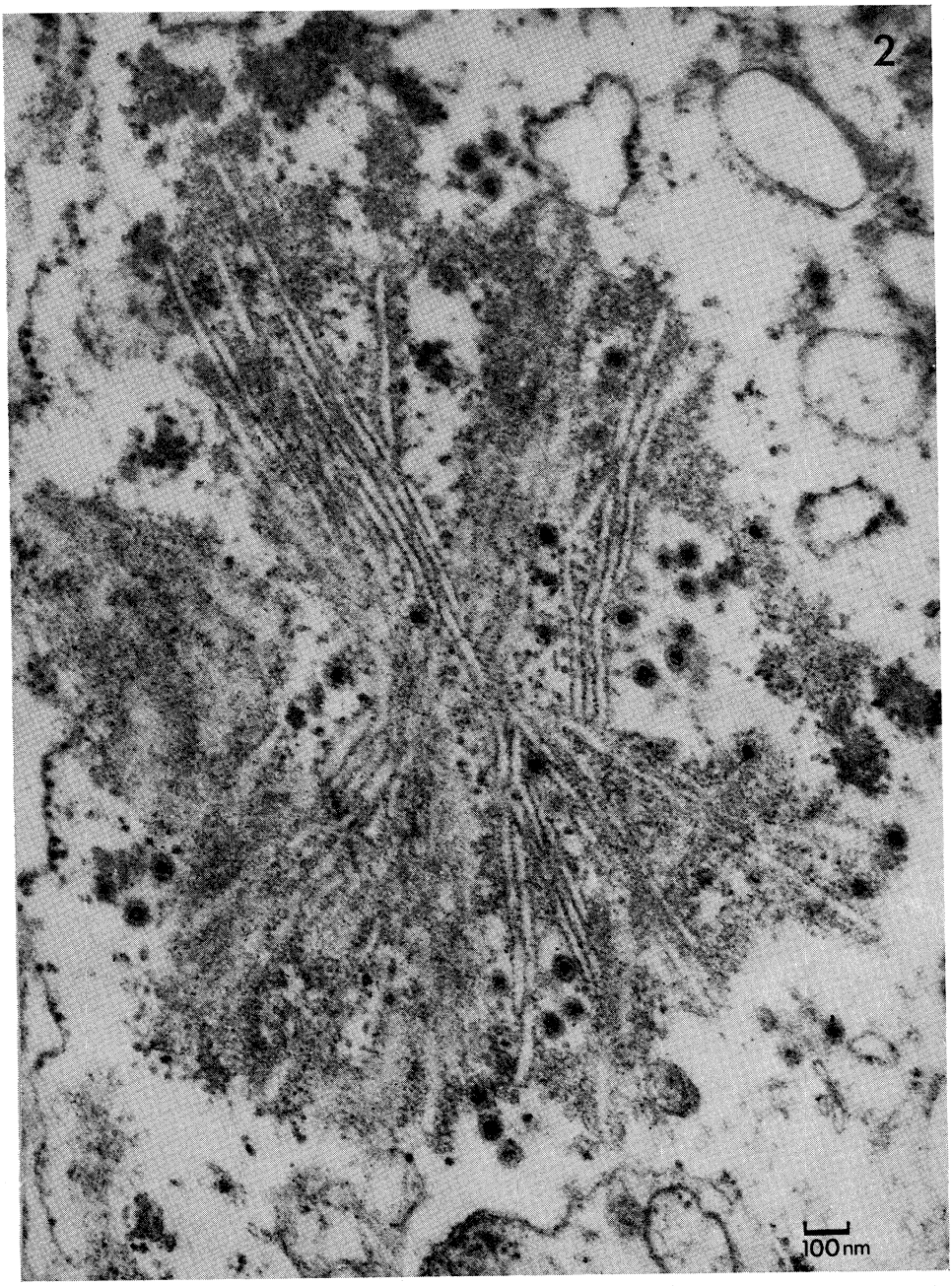


Fig. 2. Unusual structures within a fibrillogranular inclusion in the cytoplasm of a PS–EK cell infected with Tilligerry virus.

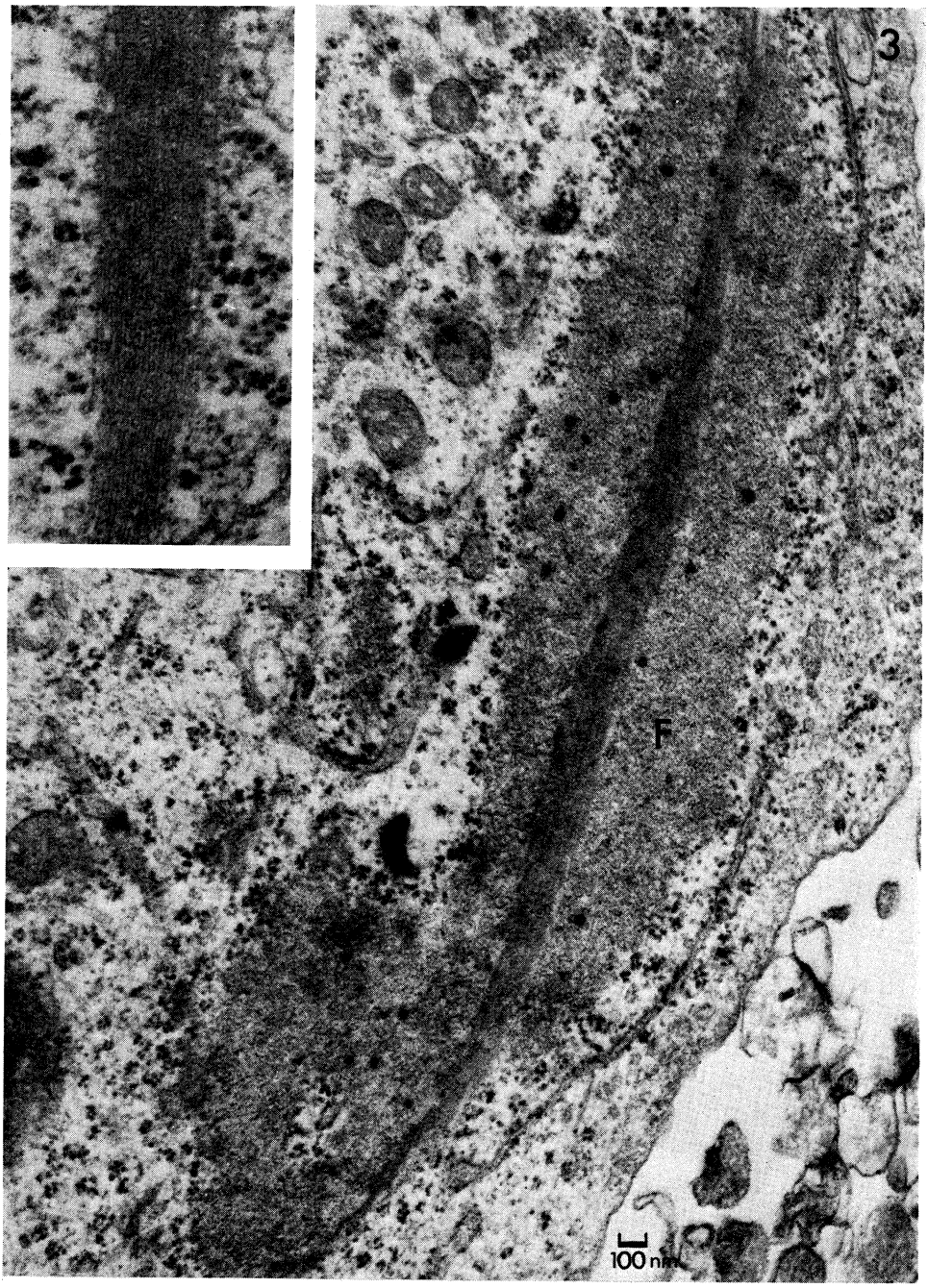
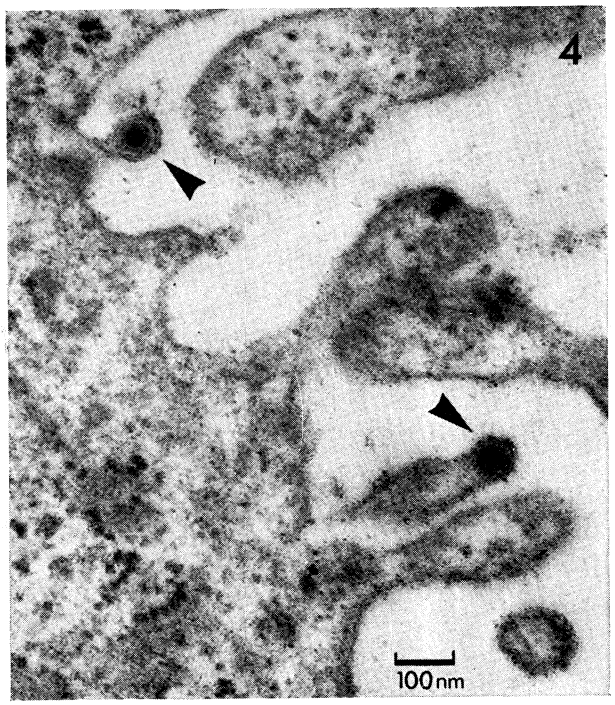


Fig. 3. An extensive striated structure within a fibrillogranular inclusion (*F*) in the cytoplasm of a PS–EK cell infected with Tilligerry virus. These structures were also seen free in the cytoplasm. × 40 000. Insert: Higher magnification to show structural detail. × 80 000.



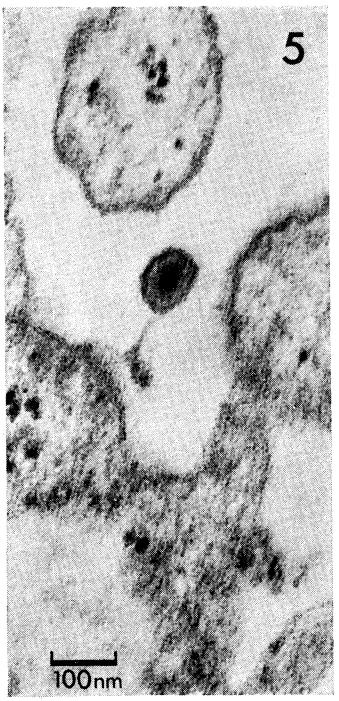


Fig. 4. Tilligerry virus particles budding from cytoplasmic processes of a PS–EK cell (arrows).
Fig. 5. Extracellular enveloped virion of Japanaut virus. PS–EK cell culture.

Gorman (1972), no negative-contrast micrographs have been included. In the case of Japanaut virus, the average diameter of the particles with an inner capsid was 57 ± 2 nm, while the diameter of the particles with an amorphous outer layer ranged from 62 to 78 nm (average 69 nm). In the case of Tilligerry virus, the particles with an inner capsid had an average diameter of 58 ± 2 nm and the diameter of particles with an amorphous outer layer ranged from 63 to 82 nm (average 71 nm).

Discussion

In both morphology and morphogenesis, Japanaut and Tilligerry viruses appear to closely resemble other orbiviruses. Not only do Japanaut and Tilligerry viruses appear to have intracytoplasmic fibrillogranular inclusions and possess a typical orbivirus capsomere arrangement on the inner capsid (Borden *et al.* 1971; Murphy *et al.* 1971), but the particles also appear to have an overall structure very similar to that determined for bluetongue virus by Verwoerd *et al.* (1972) and for seven other orbiviruses by Lecatsas and Gorman (1972). Tilligerry virus is expected to be an orbivirus because it is serologically related to the orbivirus Eubenangee (Schnagl *et al.* 1969; Gard *et al.* 1973).

A feature of the infection of cells by a number of the orbiviruses is the appearance of unusual intracellular structures. These include the filaments observed in cells infected with Colorado tick-fever virus (Murphy *et al.* 1968; Oshiro and Emmons 1968) and D'Aguilar virus (Schnagl and Holmes 1971) as well as the large paracry-

stalline inclusions seen in cells infected with African horse-sickness virus (Breese and Ozawa 1969). Intracellular structures are apparent in cells infected with Japanaut or Tilligerry viruses.

In the case of Japanaut virus it is possible that the 'coated tubules' may be basically similar to the large numbers of microtubules observed in cells infected with D'Aguilar, Mitchell River and Warrego viruses (Schnagl and Holmes 1971). The 'coated tubules' seen in reovirus-infected cells seem to be somewhat different in appearance to those in Japanaut virus-infected cells although they may be basically similar.

The extensive structures seen free in the cytoplasm and within the fibrillogranular inclusions in Tilligerry virus-infected cells (Fig. 3) could possibly be basically similar to the filamentous structures observed by Murphy *et al.* (1971) within the granular inclusions in cells infected with Tribec virus. The less frequently observed structures seen only within the fibrillogranular inclusions in Tilligerry virus-infected cells (Fig. 2), however, seem at this stage to be unique amongst the orbiviruses.

Tubular structures with a diameter approaching that of virus particles have been observed in association with the infection of cells by quite a number of orbiviruses including African horse-sickness virus (Oellermann *et al.* 1970), epizootic haemorrhagic disease of deer virus (Tsai and Karstad 1970; Murphy *et al.* 1971), bluetongue virus (Cromack *et al.* 1971; Murphy *et al.* 1971), Tribec, Wad Medani and Chenuda viruses (Murphy *et al.* 1971) and Ibaraki virus (Saito *et al.* 1972; Ito *et al.* 1973). These structures may be the result of aberrant assembly of virus capsid material, but they were not seen with either Japanaut or Tilligerry viruses.

Although the occurrence of the various orbivirus intracellular structures has been well documented, their exact connection with the respective virus growth cycles remains to be determined in almost every case, i.e. their function remains unknown.

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