Ross River Virus Replication in Cultured Mosquito and Mammalian Cells: Virus Growth and Correlated Ultrastructural Changes

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SUMMARY

The growth of Ross River virus in cultured mosquito (Aedes albopictus) and monkey kidney (Vero) cells shows similar latent periods (5 to 6 h) and maximum yields.

In Aedes albopictus cells the virus establishes a persistent, non-cytopathic infection with no significant change in cell division rate. Virus matures within large, electron-dense cytoplasmic inclusions and also at the cell membrane. Virus accumulates within the inclusions but nucleocapsids do not. ‘Type-I cytopathic vacuoles’ (Grimley, Berezesky & Friedman, 1968) are not found. Between 40 and 60 h after infection, the cell-associated virus titre falls by 1 log unit. Cytoplasmic inclusions lose electron-dense material and are transformed into microvesiculated vacuoles. Virus progressively disappears from these structures and from the cell membrane. It is suggested that during the establishment of the persistent infection, digestion of the contents of the inclusions occurs, resulting from fusion of lysosomal microvesicles with the inclusions.

In Vero cells infection leads to cell lysis. Early in infection virus is found in small cytoplasmic vesicles: ‘type-I cytopathic vacuoles’ are also present. Accumulation of nucleocapsids is marked, particularly late in infection. Thus, although the pathogenesis of Ross River virus in mice is atypical (Mires et al. 1973; Murphy et al. 1973), its ultrastructural development is similar to that of other alphaviruses in cultured vertebrate cells.

INTRODUCTION

Arboviruses are maintained in nature as a result of transmission between susceptible vertebrate hosts by haematophagous arthropods (e.g. mosquitoes, ticks). They multiply in the tissues of both their vertebrate and invertebrate host (Dalgarno & Davey, 1973). Infection of cultured vertebrate cells is in general cytopathic whereas infection of intact mosquitoes (La Motte, 1960; Thomas, 1963; Doi, Shirasaka & Sasa, 1967; Janzen, Rhodes & Doane, 1970; Whitfield, Murphy & Sudia, 1971; see, however, Mims, Day & Marshall, 1966; Rehacek, 1968), cultured mosquito cells (Filshie & Rehacek, 1968; Peleg, 1969, 1972;
Stevens, 1970) and cultured tick cells (Rehacek, 1965), generally results in a non-cytopathic, persistent infection. The underlying basis of this capacity of a virus genome to replicate in cultured cells from both homeothermic and poikilothermic organisms and to elicit such markedly different responses in each is unknown.

The short-term growth kinetics and yield per cell of an alphavirus (Semliki Forest virus, SFV) are similar in cultured mosquito and mammalian cells (Davey, Dennett & Dalgarno, 1973). A flavivirus (Kunjin) which grows more slowly and to a lower titre than SFV in mammalian cells also shows similar growth characteristics in mosquito cells.

Long-term growth experiments in cultured mosquito cells reflect to some extent the growth characteristics in the intact mosquito (Dalgarno & Davey, 1973). Titres reach a maximum at 1 to 2 days after infection and then fall gradually, stabilizing at a low level which is maintained as a persistent infection for many days (Peleg, 1969, 1972). No c.p.e. is evident even though the percentage of infective centres falls considerably from about 2 days after infection.

Two ultrastructural studies of arbovirus development have already been performed in whole insects (Janzen et al. 1970; Whitfield et al. 1971). However, in these studies the target salivary gland cells were infected asynchronously and over a long period; hence it was not possible to correlate changes in cell ultrastructure with particular stages of virus replication and the establishment of persistence.

In cultured mosquito cells infection can be at least partially synchronized. In this report we compare the kinetics of Ross River virus (RRV) growth and the ultrastructural events accompanying virus maturation in cultured Aedes albopictus and Vero cells.

METHODS

**Virus.** Ross River virus was originally isolated in Townsville, Australia by Doherty et al. (1963). The T-48 strain was obtained from Dr I. D. Marshall, John Curtin School of Medical Research, Canberra, Australia. It had been passaged 12 times in mice and 3 times in Vero cells before use. Virus stocks were prepared from Vero cells. For some experiments purified virus was used. It was prepared according to Cheng (1961) except that the pellet was resuspended in gelatin (0.2%)-saline (0.9%)-tris-HCl (0.05 M, pH 8.0) before centrifuging. Electron microscopy showed the preparation to be substantially free of non-virus contamination.

**Cells.** Aedes albopictus cells (Singh, 1967) were propagated as described by Buckley (1969). Vero cells were grown in M199/LAH medium.

**Virus growth in Aedes albopictus cells.** Cells were concentrated by centrifuging (350 g, 5 min) and 2 × 10⁷ cells suspended in 10 ml of growth medium. Cells were infected (input multiplicity, 5 p.f.u./cell) by adding either 7.5 ml of Ross River virus stock suspended in Vero cell growth medium or purified virus in gelatin-saline-tris. Adsorption was for 2 h at 30 °C with occasional gentle shaking. Cells were centrifuged at 350 g for 5 min and resuspended in 15 ml of growth medium; this procedure was repeated once. Samples (1.0 ml, 2.5 × 10⁶ cells) were distributed in loosely capped tubes and incubated at 30 °C (zero time). At appropriate times a sample (0.1 ml) was removed for electron microscopy. The remainder was centrifuged (350 g, 5 min) and the supernatant fluid and pellet were retained for extracellular virus (EV) and cell-associated virus (CAV) assay.

**Virus growth in mammalian cells.** Virus was adsorbed for 90 min at 37 °C. Monolayers were washed twice in phosphate-buffered saline and incubated at 37 °C in growth medium. Samples for electron microscopy were removed by gentle scraping.
**RESULTS**

**Growth of Ross River virus in mammalian cells**

Fig. 1A shows the growth kinetics of Ross River virus in Vero cells at 37 °C (input multiplicity, 5 p.f.u./cell); there is a latent period of approximately 6 h. At 24 h c.p.e. was evident under the light microscope.

No changes were found in the infected cells at 4 h. At 8 h intracellular enveloped virus (50 to 55 nm diam.) was present in the majority of cells examined, generally within small (0.1 to 0.3 μm diam.) cytoplasmic vesicles (Fig. 2). Membrane-limited vacuoles (0.3 to 0.75 μm diam.) enclosing attached membranous spherules (0.1 μm diam.) were also present in the cytoplasm of some cells in which virus was seen (Fig. 2, inset). It has been suggested that these structures, termed ‘type-1 cytopathic vacuoles’ (CPV-1), are sites of virus RNA synthesis (Grimley et al. 1968).

At 12 h heterogeneous cytoplasmic aggregates of electron-dense material were observed concentrated in regions of endoplasmic reticulum (results not shown). CPV-1 were present in most cells, generally 3 to 5 per cell section (Figs. 3, 4). Patches of plasma membrane with
Fig. 2. Vero cell 8 h after RRV infection. Enveloped virus is present in the cytoplasm within vesicles (arrows); nucleus (N). Inset, ‘type-1 cytopathic vacuole’ at 8 h.

attached membranous sacs (0.1 µm long) were occasionally seen (Fig. 3). No substantial accumulation of enveloped virus occurs at 12 h or at later times.

At 24 h after infection there is an increase in the number of CPV-1 and an accumulation of nucleocapsids in paracrystalline arrays in the cytoplasm (Fig. 4, inset). Occasionally nucleocapsids are associated with structures (Fig. 4) similar to ‘type-2 cytopathic vacuoles’ (CPV-2, Grimley et al. 1968). In chick cells infected with a variety of arboviruses the number of CPV-2 increases greatly late in infection (Grimley et al. 1968); this is not observed in RRV-infected Vero cells where such structures are uncommon.

**Growth of Ross River virus in mosquito cells**

Fig. 1B shows the growth kinetics of RRV in cultured *Aedes albopictus* cells at 30 °C. (The virus inoculum in this experiment was in Vero cell growth medium; see below.) Virus
Fig. 3. Vero cell 12 h after RRV infection. Note membranous sacs (S) along plasma membrane and ‘type-1 cytopathic vacuole’ (CPV).

Fig. 4. Vero cell 24 h after RRV infection. Note free nucleocapsids (arrows); some are associated with structures (heavy arrows) similar to ‘type-2 cytopathic vacuoles’ (Grimley et al. 1968); ‘type-1 cytopathic vacuole’ (CPV). Inset, paracrystalline array of nucleocapsids in Vero cell at 24 h.
Fig. 5. Cytoplasmic inclusion in *Aedes albopictus* cell 10 h after infection. Arrow indicates a nucleocapsid.

Fig. 6. Cytoplasmic inclusions with characteristic ‘halo’ in *Aedes albopictus* cell 14 h after infection.

Fig. 7. *Aedes albopictus* cell 18 h after infection with RRV. Mature virus particles (hollow arrows) are at the cell surface and within a cytoplasmic inclusion. Nucleocapsids are present in the cytoplasm (arrow).
growth shows a latent period of 5 to 6 h. The CAV titre reaches a maximum of about $10^5$ p.f.u./$10^6$ cells at 36 h and then falls over 24 h to $10^6$ p.f.u./$10^6$ cells; this level is maintained for at least a further 2 days. The EV titre continues to increase after the CAV titre starts to fall. However, between 60 and 72 h there is also a sharp decline of about 1 log unit in EV titre. This level is also maintained for a further 2 days. The constant titre over this period may reflect continued virus release since, when virus is incubated under the same conditions but without cells, the titre decreases by about 1 log unit per 24 h (M. W. Davey, D. P. Dennett & L. Dalgarno, unpublished observations).

There are no marked ultrastructural changes at 6 h after infection. At 10 h, the CAV titre has increased by one log unit and finely granular, electron-dense, cytoplasmic inclusions (1.0 to 1.5 μm diam.) are seen (Fig. 5). The inclusions appear to be enclosed by a limiting membrane which becomes more clearly defined at later stages of infection (Figs. 8 to 11). A small proportion of inclusions contains nucleocapsids (Fig. 5) identical in size to those in infected Vero cells. Occasionally nucleocapsids are found scattered free in the

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**Fig. 8.** *Aedes albopictus* cell 28 h after infection. Cytoplasmic inclusions (IC); some show loss of electron-dense material. Inset, virus budding from *A. albopictus* cell at 28 h.
cytoplasm (Figs. 7, 9). No enveloped virus is seen either within the cell or attached to the cell membrane at 10 h. Cytoplasmic inclusions were not found in mock-infected cells.

By 14 h after infection the number of cytoplasmic inclusions per cell has increased. Typically there are 5 to 6 inclusions per cell section although as many as 12 have been noted. At this time inclusions commonly show a prominent ‘halo’ of less-dense granular material (Fig. 6). Although free nucleocapsids exist in the cytoplasm, few enveloped virus particles are seen either in inclusions, free in the cytoplasm or attached externally to the cell membrane.

The first substantial accumulation of enveloped virus is noted at 18 h (Fig. 7) when the

Fig. 9. Cytoplasmic inclusion in Aedes albopictus cell 28 h after infection. A limiting membrane (circle) is evident; nucleocapsids (arrows) line the ‘cytoplasmic’ side of the inclusion membrane and are free in the cytoplasm. Most nucleocapsids in the inclusion are completely enveloped (hollow arrows).
RRV in mosquito and mammalian cells

Fig. 10. *Aedes albopictus* cell 36 h after infection. Various stages are seen in the transformation of inclusions from dense, virus-containing bodies (IC) to virus-depleted vacuolar structures containing microvesicles (V); the limiting membrane is clearly defined. Nuclear membrane (NM), nucleolus (Nu). No cell-associated extracellular virus is seen.

total (CAV + EV) titre is approximately 10⁶ p.f.u./10⁶ cells. Extracellular virus is seen at the cell membrane and intracellular virus is localized exclusively in cytoplasmic inclusions (Fig. 7). No substantial accumulation of free nucleocapsids occurs at 18 h or at later times.

Between 10 and 28 h, inclusions increase in size reaching approximately 2.5 µm in diam. (compare Figs. 5 to 8). Fig. 9 shows an inclusion at 28 h after infection. Numerous enveloped virus particles are present and proliferation of membranes is evident within the inclusion. Nucleocapsids are rarely seen within the inclusions although they line the ‘cytoplasmic’ side of the limiting membrane which is clearly visible. Some nucleocapsids appear to be budding through the membrane into the inclusion.

At 28 h, other membrane-enclosed structures containing enveloped virus were seen in the cytoplasm of infected cells (results not shown). Some bear a resemblance to the cytoplasmic inclusions; others appear as membranous sacs enclosing enveloped particles.
Fig. 11. *Aedes albopictus* cell 48 h after infection with RRV showing microvesiculated vacuolar structures (V) containing residual virus (hollow arrows). Nucleocapsids (arrows) are present in the cytoplasm. Nuclear membrane (NM), virus-containing body (IC). No cell-associated, extracellular virus is seen.

Although extracellular virus is evident at 28 h, budding of nucleocapsids at the cell membrane is seen only infrequently (Fig. 8, inset).

Between 36 and 60 h there is a sharp decline in the CAV titre. Cytoplasmic inclusions exhibit a marked loss of electron-dense material which is accompanied by the appearance within them of microvesicles and the disappearance of virus. Nucleocapsids no longer line
the ‘cytoplasmic’ side of the inclusion membrane which is now even more clearly defined. Figs. 10 and 11 show infected cells at 36 and 48 h, respectively; microvesiculation is apparent, particularly at the later time. At 36 and 48 h the cell membrane is apparently free of virus in contrast to earlier times (e.g. 18 h, Fig. 7; 28 h, Fig. 8). This is noteworthy since the CAV titre at the later times is no less, and in the case of 36 h is substantially more, than at 18 h.

No c.p.e. was noted at any stage of RRV infection: the appearance of mitochondria, nucleus, nuclear membrane and cell membrane was unaltered throughout. There was no evidence for a nuclear phase in virus development.

No significant change in cell division rate accompanied infection of Aedes albopictus cells with Ross River virus. In the experiment described, the number of cells in the infected and control cultures increased in parallel from \(2.5 \times 10^5/\text{ml}\) at zero time to \(8 \times 10^5/\text{ml}\) at 5 days. At least 50% of cells give rise to infective centres 18 h after infection; a similar proportion shows virus-specific cytoplasmic immunofluorescence (R. S. Raghow, M. W. Davey & L. Dalgarno, unpublished results). Hence ‘masking’ of either c.p.e. or a reduced cell division rate by uninfected cells is unlikely.

Aedes albopictus cells were also infected with purified Ross River virus under conditions similar to those used above. Growth kinetics were the same as those obtained when RRV was inoculated as a suspension in Vero cell growth medium and infected cells showed typical cytoplasmic inclusions. We conclude that the ultrastructural changes observed are specific to infection with Ross River virus.

**DISCUSSION**

Ross River virus development in Vero cells is characterized by (i) a limited accumulation of intracellular virus in the early stages of infection, (ii) a substantial accumulation of nucleocapsids late in infection, (iii) the presence of ‘type-I cytopathic vacuoles’, particularly late in infection, (iv) cytoplasmic disorganization and eventual cell lysis. When our results are compared with previous studies (Morgan, Howe & Rose, 1961; Acheson & Tamm, 1967; Ben-Ishai, Goldblum & Becker, 1968; Lascano, Berria & Barrera Oro, 1969; Murphy, Harrison & Collin, 1970; Murphy & Whitfield, 1970; Grimley et al. 1972) it is clear that the ultrastructure of RRV development is similar to that of other alphaviruses. This is noteworthy since the pathogenesis of Ross River virus in mice is atypical when compared with other alphaviruses (Mims et al. 1973; Murphy et al. 1973).

Ross River virus development in Aedes albopictus cells contrasts with that in Vero cells in the following ways: (i) although virus buds through the plasma membrane it also accumulates inside the cell within electron-dense, membrane-enclosed cytoplasmic inclusions, (ii) free nucleocapsids are infrequent and do not accumulate, (iii) CPV-I are not observed, (iv) the decrease in CAV titre at 36 h is accompanied by the transformation of cytoplasmic inclusions into vacuoles and multivesicular bodies, and (v) c.p.e. is not seen and the cell division rate is unchanged. However, as in Vero cells, there is no evidence for a nuclear stage in virus development.

Two previous ultrastructural studies of alphavirus development in mosquito cells examined changes in the salivary glands of infected mosquitoes (Janzan et al. 1970; Whitfield et al. 1971). Janzen et al. (1970) concluded that nucleocapsids are probably formed in the nucleus; they then pass into the cytoplasm where they attach to the ‘cytoplasmic’ side of membrane-bound vesicles and bud into the vesicle which contains numerous enveloped virus particles. They suggested that the membrane-bound vesicle releases virus from the cell by ‘reverse-phagocytosis’ but that virus may also bud directly through the plasma.
membrane. These authors detected no pathological changes within infected cells. Whitfield et al. (1971) also observed cytoplasmic vacuoles containing enveloped virus; they found no evidence of c.p.e. and no indication of a nuclear involvement in virus morphogenesis. They concluded that the essential features of virus morphogenesis in mosquito salivary glands were similar to those in mammalian cells although infection was more ‘balanced’ in the mosquito salivary gland. In neither of the studies by Janzen et al. (1970) or Whitfield et al. (1971) was it possible to clearly correlate ultrastructural changes with particular stages of virus replication due to the multi-step growth characteristics of infection in the whole insect. Although the conditions used in our study do not give a true single-cycle of virus growth, at least 50 % of the cells are infected at 18 h after adsorption (R. S. Raghow & L. Dalgarno, unpublished observations). Thus certain conclusions can probably be drawn with some confidence.

The cytoplasmic inclusions are clearly involved in the process of virus maturation. The time-course of their appearance, increase in size and loss of electron-dense material is related to the levels of cell-associated infectious virus. Free nucleocapsids are seen within inclusions early, though not late in infection (nucleocapsids are also present free in the cytoplasm). Our evidence suggests that nucleocapsids which appear in inclusions are enveloped therein, but that nucleocapsids which arise in the cytoplasm may be enveloped either on passing into the inclusion or at the cell surface. However, it appears doubtful whether virus maturing within inclusions contributes to extra-cellular infectivity unless the cell is disrupted, as in the assay for cell-associated virus. No release of individual virus particles from inclusions is seen and inclusions do not appear near the cell membrane at any stage, suggesting that ‘reverse phagocytosis’ (Janzen et al. 1970) may not occur. Further, subsequent changes in the structure of the inclusions (see below) appear to ensure that virus maturing within them is ultimately destroyed. Our results would therefore suggest that in the intact cell, infective extracellular virus primarily derives from nucleocapsids which bud directly through the plasma membrane.

Mosquito cells do not accumulate large numbers of free nucleocapsids although the level of infective virus produced is similar to that in mammalian cells. Massive accumulation of nucleocapsids occurs relatively late in infected mammalian cells and they are lost on cell lysis. In this respect, therefore, the process of infection is more ‘balanced’ in the mosquito cell (see also Whitfield et al. 1971). RRV-infected Aedes albopictus cells show an increase in CAV titre until 36 h after infection; between 36 and 60 h, the CAV titre falls by 1 log unit with no c.p.e. and no apparent change in cell division rate. In the period between 36 and 48 h, inclusions are transformed into relatively electron-lucent vacuoles and multivesicular bodies. There is a concomitant loss of virus from these structures. We interpret this to represent the digestion of the inclusion contents. This could result from the release into them of degradative enzymes present in microvesicles. We infer that the decrease in CAV titre at this time reflects these events. A similar transformation of cytoplasmic protein-storage bodies into multivesicular bodies has been observed in the fat body of uninfected insects (Locke & Collins, 1965, 1968).

One could speculate that in arbovirus-infected mosquito cells, the fall in infective centres seen after the initial rise (Peleg, 1969; R. S. Raghow & L. Dalgarno, unpublished observations) is a direct result of the digestion of virus within multivesicular bodies and vacuoles. This seems unlikely, however, since virus within inclusions probably does not give rise to extra-cellular infectivity (see above and Whitfield et al. 1971) and in any case infective centres presumably arise mainly from virus budding at the cell membrane. It is significant however, that at the time when multivesicular bodies appear, the cell membrane appears
to be completely free of virus. It may therefore be that the fall in the percentage of infective centres results from a single effect which is reflected in two apparently separate responses.

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