Replication and morphogenesis of *Amsacta moorei* entomopoxvirus in cultured cells of *Estigmene acrea* (salt marsh caterpillar)

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A study of the sequence of morphogenic events occurring within *Amsacta moorei* entomopoxvirus-infected *Estigmene acrea* cells is presented. Stages in virion development, and the various cytopathic effects observed in these cells between 0 and 120 h post-infection (p.i.) are described. Events in the early stages of virion assembly (24 to 48 h p.i.), the formation of the outer viral membrane (48 to 72 h p.i.) and the development of occlusion bodies or spheroids (72 to 120 h p.i.) were identified. Cells grown in TC100 culture medium supported production of mature virus particles, the majority of which were either of the intracellular naked virion form, or mature virions incorporated into occlusion bodies. Only limited production of the extracellular enveloped form was observed in these cells.

The *Amsacta moorei* entomopoxvirus (AmEPV) is one of the most well studied members of the subfamily Entomopoxviridae. It is currently classified with the intermediate-sized entomopoxviruses (genus B) that have been isolated from Lepidoptera and Orthoptera (Matthews, 1982). In common with the other entomopoxviruses (Bergoin *et al.*, 1969; Goodwin & Filshie, 1969; Henry *et al.*, 1969) and some vertebrate poxviruses (Ichihashi *et al.*, 1971), AmEPV produces three forms of virion, the non-occluded intracellular naked virion, the extracellular enveloped virion (EEV) and occluded virions. The spheroid occlusion bodies are primarily composed of spheroidin protein (Langridge *et al.*, 1977; Langridge & Roberts, 1982; Hall & Hink, 1990). The spheroidin gene has been identified and sequenced, and encodes a polypeptide of 114 K (Hall & Moyer, 1991). Spheroidin forms a crystalline matrix which protects the mature virions during transmission between insects (Goodwin *et al.*, 1990; Granados & Roberts, 1970).

Relatively little is known about the AmEPV replication cycle, although cell lines from *Lymantria dispar* (Goodwin *et al.*, 1990), *Heliothis zea*, *Spodoptera frugiperda*, *Bombyx mori* and *Estigmene acrea* (Granados & Naughton, 1975; Granados, 1981) have been reported to support virus replication. From observations made in AmEPV-infected *E. acrea* larval tissues at 9 and 14 days post-infection (p.i.), various stages in virion maturation have been identified (Granados & Roberts, 1970). Because these infections were asynchronous, a sequence of virion morphogenesis was proposed using the vertebrate poxviruses as a model (Bergoin & Dales, 1971). These studies identified primary sites of virus assembly within distinct regions of the cytoplasm adjacent to the nucleus, termed viroplasms and designated type I or II depending upon the arrangement and composition of the granular material contained within. Immature virus particles that formed in the viroplasms were shown to be formed from sections of membrane, which circularized and filled with nuclear material. This condenses during virus maturation to form a characteristic 'brick-shaped' core. In cross-section, the core was observed to contain rod-like structures, suggesting that the nuclear material forms a continuous, filamentous coiled rod (Granados & Roberts, 1970). The outer virus membrane, of both naked and occluded virions, has been shown to possess a beaded surface (McCarthy *et al.*, 1974) similar to that observed in *Melolontha melolontha* EPV (Bergoin *et al.*, 1971) and vaccinia virus (VV) (Dales, 1963; Medzon & Bauer, 1970; Dales & Pogo, 1981).

In this study, AmEPV-infected *E. acrea* cells at times from 0 to 120 h.p.i. were examined using transmission electron microscopy (TEM), and an ordered sequence of morphogenic and cytopathic events was established in cultured cells.

*E. acrea* cells (EAA-BTI) were kindly supplied by Dr W. F. Hink (Ohio State University Columbus, Ohio, U.S.A.). Cells were grown and infected as previously described (Marlow *et al.*, 1992), and were examined under the light microscope from 24 to 96 h.p.i. for cytopathic signs of infection. All samples were fixed in a mixture of 2.5% glutaraldehyde/2% paraformaldehyde, post-fixed in 1% osmium tetroxide, washed several times in cacodylate buffer and then dehydrated through a graded alcohol series before infiltration with Spurr's resin. Ultrathin sections were stained with uranyl acetate and lead citrate, and were examined by TEM.
Control, uninfected *E. acrea* cells produced mono- or bipolar processes. Following infection with AmEPV the cells rounded up between 12 and 24 h.p.i., and by 48 h.p.i. the cytoplasm was visibly grainy and highly vacuolated as previously described (Marlow et al., 1992). Between 96 and 120 h.p.i., infected cells contained varying numbers of spheroids, with an estimated 95% of the cells containing mature spheroids at 120 h.p.i.

By using a high multiplicity of infection (m.o.i. = 20), we were able to achieve synchronous infection of the cells so that the sequence of morphological events during virus maturation could be ordered with respect to time. Various stages in the replication cycle of AmEPV that were observed confirmed previous findings (Goodwin et al., 1990), but additional intermediate stages in virion assembly between 48 to 72 h.p.i. were demonstrated.

The first signs of virus infection at 24 h.p.i. were the appearance of discrete type I viroplasms within the cytoplasm, often adjacent to the nucleus (Fig. 1a). These electron-dense areas, previously described by Granados...
Fig. 2. Intermediate stages of virus morphogenesis. (a) Immature virus cores in association with cytoplasmic vesicles (V) become enveloped in a double membrane. (b) The outer membrane acquired between 48 and 72 h p.i. is formed from short curved sections (arrowheads), in several layers around the core (im). Polyribosome-like structures were often present in association with the assembling particles (arrows). (c) Core maturation proceeds during acquisition of the outer membrane (arrow), which is cytoplasmic in origin (arrow); the core membrane has an outer spicule-coated layer (arrowheads). Bar markers represent 0.1 μm. (d) The outer membrane appears to consist of several layers, with distinct subunits undergoing differentiation. Cytoplasmic fibrils are present, associated with assembling virions (cf). Bar marker represents 0.5 μm.

& Roberts (1970) in infected larval tissue, are the sites of DNA replication and early virion assembly events, by analogy with VV-infected cells (Bergoin & Dales, 1971).

Between 12 and 24 h p.i., crescent-shaped membranes began to appear within, and at the periphery of, the viroplasms and filled with viroplasmic material to form immature viral particles. The majority of these particles appeared to form within the first 24 h after infection, but were also present in cells examined at 48 h p.i., in close association with the viroplasmic areas. In addition, at 48 h p.i. multiple membranes were observed around some of the cores (Fig. 1 b). Infected cells sampled at 72 (Fig. 1 c, d) and 96 (Fig. 1 e) h p.i. contained various forms of virus particle. Although the immature forms were still present at 72 h p.i., many particles had acquired an outer membrane (intermediate assembly stage) (Fig. 1 c, d). The section in Fig. 1 (d) shows the characteristic beaded surface of this outer membrane. At 72 and 96 h p.i., the cells contained characteristic bundles of fibrils and developing occlusion bodies. The sections of the outer membrane were cytoplasmic in origin, and were often found in association with cytoplasmic vesicles (Fig. 2 a). As with VV and other EPVs, the membrane is probably derived from the Golgi apparatus. The mem-
brane formed several layers around the core (Fig. 2b), and this process may correspond to the second wrapping stage in VV assembly (Dr G. Griffiths, EMBL, University of Heidelberg, Germany, personal communication). This second membranous wrapping of the virus core appeared to proceed simultaneously with the maturation events occurring within the core between 72 and 96 h p.i. (Fig. 2b to d). The outer membrane complex appeared to be composed of a unit membrane (Fig. 2d), with distinct subunits, undergoing differentiation. The area between the core and the outer membrane, shown in Fig. 2(c), may be the site of lateral body development. Similar membrane structures have also been observed in AmEPV-infected Galleria mellonella larvae (Roberts, 1970), but their function was not discussed. Our observations suggest that these membrane complexes form the outer viral envelope, and may undergo differentiation to give rise to the beaded surface morphology commonly observed in negatively stained virion preparations (McCarthy et al., 1974). The transverse section in Fig. 2(c) shows the core surrounded by a double membrane with a smooth inner layer and an outer, spicule-coated layer. Polyribosome-like structures were often observed in association with the assembling virions (Fig. 2b), and occlusion bodies in AmEPV-cells were similar to those found by Ichihashi & Dales (1973) in cowpox virus infections, and are probably involved in the assembly process.

As in previous reports (Dales, 1963; Bergoin et al., 1971; Granados, 1973; Kurstak & Garzon, 1977; Goodwin et al., 1990), a number of cytopathic effects were observed in infected cells between 72 and 96 h p.i. These included the presence of spherical, para-crystalline bodies within the nucleus (Fig. 1c), and bundles of cytoplasmic fibrils which often appeared to be associated with immature virus particles (Fig. 1c, d). The latter may be modified host cell cytoskeleton proteins, such as actin, or tubulin, which have been implicated in the assembly process of poxviruses (Hiller et al., 1979). This is currently being investigated further using immunolabelling techniques.

The routinely low titres ($5 \times 10^6$ TCID$_{50}$ maximum) of extracellular virus produced by AmEPV-infected E. aerea cells may be partially explained by the retention of large numbers of mature virus particles within the cytoplasm, as the intracellular naked form (Fig. 3a). Although many of these were observed at the periphery of the cell, there was little evidence of exocytic release. This may also partially explain the difficulties involved in obtaining multiple rounds of infection when using a low m.o.i., and in obtaining a reliable plaque assay in E. aerea cells. Virions were released passively from the cell after 144 h p.i., following disintegration of the plasma membrane (not shown). Virus released passively fol-

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Fig. 3. Intracellular maturing virus particles. (a) Cells contained virions grouped close to the plasma membrane (pm). (b) Early nucleation of virions at 72 h p.i., showing the maturing virions during occlusion into the spheroidin protein. (c) Spheroid development at 120 h p.i., containing numerous particles, with the characteristic halo around the mature particles. Bar markers represent 0.5 μm.
lowing breakdown of the plasma membrane, or via artificial cell disruption, will not acquire the outer envelope derived from the plasma membrane. In VV, the outer envelope contains Vp37 (Blasco & Moss, 1991), a glycoprotein important for cell-to-cell transmission. In one of the alternative cell lines, L. dispar, that supports AmEPV replication, extracellular enveloped virions are produced (C. P. Palmer, S. A. Marlow & L. A. King, unpublished) and provide a reliable plaque assay and secondary infection (Goodwin et al., 1990; Hall & Moyer, 1991). This would suggest that the cell line appears to influence the form of virion produced.

Between 72 and 96 h p.i., nucleating groups of mature virions became occluded into the spheroidin protein in a random arrangement (Fig. 3b, c). The immature spheroids were small and round, but as they matured (Fig. 3b) they adopted the characteristic oval shape (Fig. 3c). Virions undergoing occlusion at 72 h p.i. exhibited structural features characteristic of the mature virions, suggesting that occlusion of mature virions occurs, as well as post-occlusion virion maturation, as has been previously reported (Granados, 1981).

In conclusion, the series of events observed in the maturation of AmEPV virions in vitro appear to be closely related to that reported for VV (Dales, 1963; Dales and Kajikoa, 1964; Ichihashi et al., 1971; Dr G. Griffiths, personal communication) and confirm most of the maturation stages proposed by Granados & Roberts (1970) from their in vivo replication studies.

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References


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