Morphological Observations on the Replication of Herpesvirus saimiri in Monkey Kidney Cell Cultures

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SUMMARY

Owl and African green monkey kidney cell cultures have been infected with 1 p.f.u./cell of herpesvirus saimiri and sample cultures have been taken for examination by electron microscopy at 3 to 6 hourly intervals over a period of 7 days; the experiments were repeated several times. The peculiarly slow replication cycle of Herpesvirus saimiri has enabled distinct cytoplasmic and nuclear phases in virus maturation to be clearly distinguished; the overall fine structural features were similar in both cell types. Immature particles were first detected in the nucleus and cytoplasm 63 h after infection. Thereafter, abundant cytoplasmic immature particles matured by budding through cytoplasmic membranes until about 100 h, whereas nuclear immature particles budded through the inner nuclear membrane or intranuclear invaginations of it later, from about 100 h until cytolysis was complete at 160 h. Morphological differences were also observed between particles budding at cytoplasmic membranes and the nuclear envelope. At the former site the membrane overlying the bud showed an electron opaque thickening which imparted to the mature particle an asymmetrical appearance. Such thickenings of the envelope were not observed in mature particles of nuclear origin.

Unusual tubular and laminated nuclear structures were seen towards the end of the replicative cycle corresponding with the phase of nuclear virus maturation by budding; the morphology of the latter structures is described.

INTRODUCTION

Herpesvirus saimiri (HVS) is of special importance since it was the first member of the herpesvirus group found to be oncogenic in sub-human primates (Meléndez et al. 1969a, b; Morgan et al. 1970). The structure of the agent was determined originally by examining cultures harvested at the time of maximum c.p.e. (Morgan et al. 1970). In such material, preliminary observations suggested that virus maturation by budding through cellular membranes occurred both in the cytoplasm and at the nuclear envelope (Morgan et al. 1970).

In subsequent morphological studies, cultures showing c.p.e. were examined on single occasions a considerable time after infection (more than 5 days) and two surprising findings were described: (1) that cytoplasmic maturation of the virus was extremely rare and (2) that the virus matured in the nucleoplasm by becoming enveloped in newly synthesized membranes laid down around individual ‘incomplete’ particles (Heine, Ablashi & Armstrong, 1971), a mechanism not hitherto known for any herpesvirus. Further
electron microscope observations on HVS-infected cells have not shed light on these points (King et al. 1972) and it was decided therefore to follow the fine structural aspects of the growth and maturation of HVS throughout its replication cycle.

METHODS

Cells and technique of culture. The owl monkey kidney (OMK) and African green monkey kidney (AGMK) cells and culture techniques used were exactly the same as for earlier work (Morgan, Achong & Epstein, 1973). For any one set of experiments cultures were used at an identical passage level from the same stock bottle.

Virus. Stock virus pools were prepared from infected 2-litre glass roller bottle cultures of AMGK or OMK cells showing advanced c.p.e. and were stored at $-70 \, ^\circ\text{C}$ as previously described (Morgan et al. 1973). Samples were assayed in OMK cells by the plaque titration first developed for HVS by Daniel et al. (1971), using 2.5% methyl cellulose (Methocell, The Dow Chemical Company, Michigan, U.S.A.) as the overlay. Plaques were counted after 6 days of incubation and titres expressed as p.f.u./ml. The titres were usually in the region of $2 \times 10^6$ p.f.u./ml.

Infection of cultures. Experimental cultures were always inoculated with 1 p.f.u./cell. The inoculum was left at $37 \, ^\circ\text{C}$ for 1.5 h to allow virus adsorption and was then removed by gentle washing of the cell sheet with phosphate buffered saline. The appropriate volume of maintenance medium was then added.

Electron microscopy. Cells were detached from the surface of Falcon flask cultures by a combination of flushing and gentle scraping with a bent Pasteur pipette, and were suspended in about 1 ml of the medium in which they were grown. The cell suspension was fixed, pelleted by centrifugation, dehydrated, and embedded in epoxy resin, and sections were cut, stained and examined as in previous work (Morgan et al. 1973).

Light microscopy. Stock cultures were checked routinely before use in a Reichert inverted microscope. Infected cultures were examined in a similar manner throughout each experiment.

Experimental procedure. In a first set of experiments, infected Falcon flask cultures of AGMK and OMK cells were harvested and prepared for electron microscopy at 12 hourly intervals until complete cytolysis, and an outline of the salient morphological steps in virus replication was obtained. The cultures were also examined by light microscopy at the same time intervals to follow the progress of c.p.e.

In a second set of experiments similar cultures were harvested for fine structural study every 6 h after the first appearance of virus in the cells and in addition, 3 hourly during the period of virus maturation in the cytoplasm as revealed by the first experiments.

Uninoculated cell cultures of the appropriate cell type were set up in each experiment and were followed by light microscopy and sampled for electron microscopy at the mid-point and end of the virus replication cycle.

RESULTS

General observations

Infection of OMK and AGMK cultures at the rate of 1 p.f.u./cell produced discrete cytopathic foci of rounded cells in the monolayers 3 to 4 days later (Fig. 1). Thereafter, infection extended gradually throughout the culture (Fig. 2) destroying the cell sheet within 6 to 8 days. Four experiments with OMK cells and three experiments with AGMK cells
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Fig. 1. Photomicrograph of a live African green monkey kidney cell culture 3½ days p.i. with Herpesvirus saimiri seen by oblique illumination. An early focus of c.p.e. involving about 15 clumped, rounded cells is surrounded by a monolayer of intact cells. Magnification ×90.

Fig. 2. Photomicrograph of a live African green monkey kidney cell culture 6½ days p.i. with Herpesvirus saimiri. The monolayer is almost completely destroyed with rounded, degenerate cells present among sparse normal cells. Magnification ×90.

Fig. 3. Electron micrograph showing detail of nucleus from an owl monkey cell infected for 78 h with Herpesvirus saimiri; part of the nuclear envelope can be seen in the lower left corner of the field. The nucleoplasrn contains immature particles which are either empty (long arrows) or have central ring-shaped (short arrows) or dense nucleoids.
each involving sequential sampling of infected cultures throughout the replicative cycle, indicated that the overall fine structural appearances of virus maturation were remarkably similar in both systems except for clear-cut differences in one aspect of virus maturation and in the arrangement of unusual membrane structures which developed in the nucleus; these differences are described below.

**Electron microscope observations**

**First detection of virus**

Immature particles were detected for the first time in the nucleus and cytoplasm of both OMK and AGMK cells 63 h post infection (p.i.). Thereafter, the immature particles continued to be present in the nucleus right through to the end of the replicative cycle at 160 h (Fig. 3, 4, 6 to 9), but were abundant in the cytoplasm only up to 112 h in both cell types (e.g. Fig. 4 and 5). The immature particles in the cytoplasm invariably possessed a dense central nucleoid (Fig. 4 and 5), whereas intranuclear particles included, in addition, forms which were either empty or contained a ring-shaped structure in the centre (Fig. 3, 6 to 9).

**Virus maturation**

In every experiment maturation of immature particles by budding through cellular membranes was first observed in the cytoplasm. Budding into cytoplasmic spaces (Fig. 4 and 5) with accumulation of mature particles within, was seen in both OMK and AGMK cells, but budding at the plasmalemma, evidenced by the impressive accumulation of mature particles adjacent to the cell surface in intercellular spaces (Fig. 6), was only observed in OMK cells. Mature enveloped particles of cytoplasmic origin were first detected at 63 h and became increasingly frequent thereafter, reaching a peak in the extracellular space about 100 h p.i. In contrast, maturation by the budding of intranuclear particles through the inner nuclear membrane (Fig. 7) or into elaborate invaginations of this structure within the nucleoplasm (Fig. 8 and 9) did not commence until about 100 h and reached maximum activity between 124 h and 136 h (Fig. 9). Throughout the period of maturation the nuclear membrane and other membrane-bounded compartments of the cell remained intact (Fig. 3 to 9) and it was only towards the end of nuclear maturation after 132 h that breakdown of membranes developed, followed by lysis of the cell (140 to 160 h). The association of lysis with virus maturation in the nucleus was further reflected by the repeated finding of abundant immature and mature particles in the nuclear debris of disintegrated cells in which cytoplasmic particles were rare.

**Fine structure of mature particles**

In OMK and AGMK cells morphological differences were observed between mature particles formed in the cytoplasm and those which developed in the nucleus. These differences arose from structural alterations to cellular membranes at the site of virus budding. Cytoplasmic membranes overlying budding immature particles showed increased electron density in the area of the bud (Fig. 4 and 5) which, as maturation progressed, formed a crescent-shaped mass giving the particles an asymmetrical appearance. As a result, mature particles in cytoplasmic vacuoles (Fig. 5) and in the extracellular space (Fig. 6) were elliptical in outline and measured about 175 nm by 140 nm across. Mature particles with damaged or incomplete envelopes were often seen in these two locations and in some instances only the crescentic, thickened portion of the outer envelope remained (Fig. 6). In contrast, structural changes were never observed in nuclear membranes at the
Fig. 4 to 10 are electron micrographs of thin sections of cultured monkey kidney cells harvested at intervals after infection with Herpesvirus saimiri, fixed in glutaraldehyde, embedded in epoxy resin and stained with uranyl acetate.

Fig. 4. Owl monkey cell 84 h p.i. The cytoplasm above is separated from the nucleus below by an intact nuclear membrane running obliquely across the field; immature virus particles are seen in both nucleus and cytoplasm. All those in the cytoplasm contain a dense central nucleoid and some are in the process of budding through cytoplasmic membranes (arrows). The membranes overlying budding particles show crescentic thickening and increased electron density.

Fig. 5. Detail of immature particles with dense nucleoids budding into cytoplasmic spaces of an owl monkey cell 90 h p.i. Part of the nucleus with clumped marginated chromatin is seen on the left; the intact nuclear envelope has been sectioned obliquely. There is an electron-dense, crescentic thickening of cytoplasmic membranes overlying the site of budding (arrows).
Fig. 6. Elliptical mature particles with an eccentric crescent of electron-dense material in the envelope are seen lying free in the intercellular space between adjacent owl monkey cells at 108 h p.i. The nucleus of the lower cell, with its intact envelope traversing the field, contains immature particles which are either empty or with central ring-shaped or dense nucleoids. Most of the extracellular particles have a ragged, damaged envelope with, in some cases, only a crescentic thickened portion remaining.

Fig. 7. Parts of a lobed nucleus of an owl monkey cell infected 96 h previously. An immature particle is seen (right) at an advanced stage of budding through an intact inner nuclear membrane into the perinuclear space. The membrane enveloping the particle does not show the crescentic thickening evident in cytoplasmic viral budding (cf. Fig. 4 and 5). Three other immature particles are also present in the nucleoplasm.
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Fig. 8. Owl monkey cell nucleus 108 h p.i. with the intact nuclear envelope curving across the top right side of the field. Mature particles can be seen lying within intranuclear membrane-bounded spaces formed from invaginations of the nuclear membrane. These mature particles, like all others budding at nuclear membranes (cf. Fig. 7 and 9), are free of the localized crescentic thickening in the envelope associated with budding at cytoplasmic membranes (cf. Fig. 4 to 6). Immature particles are also present in the nucleoplasm.

Fig. 9. Detail of African green monkey cell 136 h p.i. with cytoplasm above and the nuclear envelope crossing the top of the field. Groups of mature particles are seen lying within intranuclear invaginations of the perinuclear space. The membrane limiting the invaginations is well seen where it has been cut transversely (arrows) and its continuity with the inner nuclear membrane is evident at X. Immature particles are also present in the nucleoplasm.
Fig. 10. Details of owl monkey cell nucleus with 2 laminated structures cut in various tangential and longitudinal planes. Each structure consists of a stack of electron opaque laminae separated from one another by 25 nm wide electron lucent zones; the electron-dense particles in the centre of these zones are well seen where the section has cut the stack perpendicular to the surface. The stack on the left is unusual since it consists of an array of 14 separate laminae. An immature particle with a ring-shaped nucleoid shows some connection with the outermost lamina of the right hand stack; the electron-dense material appears to be continuous with the capsid (arrow) whilst the electron-dense particles are associated with the ring-shaped nucleoid.

site of virus maturation by budding (Fig. 7) and in consequence, mature particles of nuclear origin were round and regular, measuring 140 nm in diam. (Fig. 8 and 9).

Unusual virus-associated nuclear structures

Besides the intranuclear tubular structures seen in both OMK and AGMK cells at the time of virus maturation by budding through nuclear membranes, and described in detail elsewhere (Morgan et al., 1973), the nuclei of infected OMK cells also contained elaborate layered arrays (Fig. 10) consisting of parallel stacks of electron opaque laminae separated from one another by an electron-lucent zone 25 nm wide having a line of electron-dense particles running down the centre. Most of these structures contained a series of about four or five of the electron-dense laminae but in some cases as many as fourteen were encountered (Fig. 10). In several cases the outermost lamina was seen to be continuous with immature particles at the stage with a ring-shaped nucleoid (Fig. 10).
DISCUSSION

The findings reported here confirm and extend those of earlier work on the morphology of HVS replication (Morgan et al. 1970) and disagree in at least two important respects with the results of Heine et al. (1971) using similar monkey kidney cell systems. Thus, from 63 to 112 h in both AGMK and OMK cells, budding through cytoplasmic membranes predominates (Fig. 4 and 5), whereas budding through the nuclear membrane (Fig. 7) occurs at a later stage between 100 h and the end of the replicative cycle. It is not surprising that this important cytoplasmic phase of HVS maturation was missed in previous ultrastructural studies (Heine et al. 1971; King et al. 1972) since these were not based on closely spaced sequential sampling of the kind used in the present work. Indeed, the observations of Heine et al. (1971) were made on single samples taken from infected cultures long after this phase was over (more than 5 days p.i.). Furthermore, the present extensive observations have failed to yield evidence of virus envelopment in the nucleus by the de novo accumulation of membrane material around the immature particle as claimed by Heine et al. (1971). It might be that the elaborate infoldings of the nuclear membrane seen during the second half of the replicative cycle (Fig. 8 and 9) were mistaken for such de novo synthesized intranuclear envelope material (Heine et al. 1971), particularly since the single samples examined were prepared by flat embedding.

The elegant circular symmetry of mature particles in the perinuclear space (Fig. 8 and 9) in contrast to the elliptical, eccentric and ragged appearance of cytoplasmic and extracellular particles (Fig. 5 and 6) calls for comment. Since many of the cytoplasmic and extracellular mature particles also showed a focal breakdown of the envelope with exposure of the underlying capsid it is conceivable that the whole appearance might be the result of mild hydrolytic digestion. It has long been shown that primate kidney cells contain a profusion of lysosomes (Maunsbach, 1969) and it could be expected that traces of newly synthesized lysosomal enzymes are therefore present within membrane-bounded cytoplasmic spaces. Such enzymes from disrupted cells must also accumulate in the intercellular spaces and might alter the morphology of the mature particle.

With regard to the unusual HVS-associated intranuclear structures, it has already been pointed out that the tubules appear to be composed of capsids apparently assembled in linear arrays (Morgan et al. 1973) and the intimate connection of immature particles with the laminated stacks reported here (Fig. 10) suggests that these, too, may represent an aberrant assembly of virus components.

Although the gradual nature of HVS replication in monkey kidney cells has emphasized the importance of the cytoplasm in the growth and maturation of this virus and by analogy, possibly also for other members of the herpesvirus group whether connected with oncogenesis or not, several questions remain regarding the sites of assembly and accumulation of virus structural components and their subsequent transport within the cell. In particular, it is not clear whether immature particles are assembled in the cytoplasm and if not, how they reach this region from assembly sites in the nucleus of cells whose membrane-bounded compartments are manifestly intact (Fig. 3 to 9); for a phase of passage from the nucleus to the cytoplasm has not been found despite the close sampling (3 hourly) carried out throughout the critical period. The present findings using the HVS system with its advantageous slow replication cycle has further emphasized the long recognized cytoplasmic element in herpesvirus morphogenesis (Epstein, 1962; Epstein & Holt, 1963; Lunger, Darlington & Granoff, 1965; Epstein et al. 1965, 1968; Achong & Meurisse, 1968) but at the same time has heightened the discrepancy between the findings of fine structural
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