Acta virol. **25** : 101—107, 1981

ELECTRON MICROSCOPY OF RABBIT EMBRYO FIBROBLASTS INFECTED WITH HERPESVIRUS ISOLATES FROM *CLETHRIONOMYS GLAREOLUS* AND *APODEMUS FLAVICOLLIS*

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Received November 27, 1980

Summary. — Electron microscopy of rabbit embryo fibroblasts infected with five herpesvirus isolates from murine rodents *(Clethrionomys glareolus* and *Apodemus flavicollis)* revealed morphological changes characterized by the formation of intranuclear vacuoles, in which envelopment of nucleocapsids took place. The herpesvirus nucleocapsids were formed in the vicinity of intranuclear granular inclusions. Margination of chromatin, disaggregation of nucleoli and, in some isolates, the occurence of tubular structures with a diameter of 33—35 nm characterized the ultrastructural changes in the nuclei. In the cytoplasm, membraneous vacuoles, containing numerous naked nucleocapsids and, in some isolates, large electron-dense bodies, containing non-enveloped nucleocapsids, were formed. Extracellular spaces contained either enveloped virions which, besides nucleocapsids, contained in their envelope a part of the dense material from intracytoplasmic dense bodies, or viral envelope-like structures without nucleocapsids partially filled with dense material from intracytoplasmic dense bodies. Ultrastructural development of these isolates in REF resembled that of freshly isolated herpes simplex type 1 viruses but some morphological changes resembled those characteristic of cytomegaloviruses.

Key words: Herpesviridae; free-living rodents; rabbit embryo fibroblasts; electron microscopy

Introduction

Electron microscopy of the growth cycle in cell cultures of either freshly isolated or laboratory-maintained strains revealed great differences among members of the family Herpesviridae.

Freshly isolated strains of human herpesvirus type 1 (HSV 1) (Nii *et al.*, 1968; Rajčáni *et al.* 1975, 1977; Iwasaka *et al.* 1978; and others) in HEp 2 cell cultures form numerous crystaline arrays but relatively few cytoplasmic

structures containing enveloped nucleocapsids. On the other hand, laboratory-maintained strains are characterized by numerous cytoplasmic structures with enveloped nucleocapsids and only relatively few crystalline arrays of nucleocapsids in cell nuclei. Fresh isolates of HSV 1 form numerous filamentous structures in cell nuclei, while unenveloped or partially enveloped nucleocapsids occur in the cytoplasm (Schwartz and Roizman, 1969). Similarly, electron microscopy of FL cells infected with freshly isolated HSV 1 strains (Iwa, Ko and Watanabe) revealed the formation of structures (dense bodies, tubular structures in the nuclei) which, according to their morphology, rather resembled some steps of the morphological development of cytomegaloviruses in infected cells (Nii, 1971*a*, *b*; Nii and Yasuda, 1975, 1976).

Cytomegalovirus (CMV) has a unique replication cycle among herpesviruses, characterized by the formation of intranuclear and intracytoplasmic structures and viral inclusions (Middelkamp *et al.*, 1967; Haguenau and Michelson-Fiske, 1975; Garnett, 1979; Fong *et al.*, 1980).

The aim of our study was to compare the ultrastructural changes in rabbit embryo fibroblasts (REF) at intervals after infection with five freshly isolated but not definitely classified, herpesviruses from free living rodents: the bank vole *Clethrionomys glareolus* and the yellow-necked mouse *Apodemus flavicollis* (Blaškovič *et al.*, 1980).

Materials and Methods

Viruses and cells. Five isolates (Nos 60, 68, 72, 76 and 78) were recovered from pools of different organs of Clethrionomys glareolus and *Apodemus flavicollis* (Blaškovič *et al.*, 1980). REF were used as the most suitable host cells for herpesvirus infection. This stable cell line was derived by Dr. B. Řezáčková, Institute of Sera and Vaccines, Prague. The cells were grown in basal Eagl medium (BEM) with 5 % heat-inactivated calf serum.

Electron microscopy. REF grown in Roux bottles were infected with the respective five herpesvirus isolates at multiplicities of infection (MOI) of 1 or 0.1 and incubated for 60 hr and 5 (7) days, respectively. The cells were then scraped off from the glass with a rubber policeman and pelleted by centrifugation at 2000 rev/min for 15 min. The pellet was fixed with 2.5 % glutaraldehyde in 0.2 M sodium cacodylate buffer, pH 7.2, for 30 min at 4 °C. After repeated short washing with cacodylate buffer, the cell pellet was post fixed in 1 % osmium tetroxide in cacodylate buffer at pH 7.2 for 60 min at room temperature. The fixed cell pellet was dehydrated in increasing concentrations of acetone and embedded in epoxide resine Araldit CY 212 (Serva, Heidelberg). Ultrathin sections cut on an Ultrotome LKB III ultramicrotome were stained with a 2 % aqueous solution of uranyl acetate and lead citrate (Venable and Coggeshall, 1965). Specimens were examined in a Philips EM 300 electron microscope at 80 kV.

Results

REF infected with the five herpes virus isolates at a MOI of 1 were examined at 60 hr after inoculation, when approximately 80% of the cells became detached from the glass and the remaining cells were rounded. REF infected at a MOI of 0.1 were examined after 5 days, when 50% of the cells became detached from the glass or after 7 days, when approximately 80% of the cells became detached from the glass.

The individual herpesvirus isolates caused in REF identical morphological changes connected with virus reproduction at the given interval after inoculation.

Ultrastructural changes in the nuclei of infected cells

The nuclei of infected cells showed, besides margination of chromatin which is a general characteristic of herpesvirus infection of cells, disaggregation of nucleoli and formation of numerous lobes of nuclear membrane. Very characteristic was the formation of irregular granular structures connected with naked nucleocapsids, often localized close to the nuclear membrane (Fig. 1)., as well as the formation of diffuse finely granular structures also connected with the formation of naked nucleocapsids. Close to the structures with a granular substructure, capsids with small electron opaque core, capsids with densely staining electron opaque core and empty electron-translucent capsids were observed (Fig. 2). In contrast to some known morphological changes accompanying the HSV and CMV infection, the nuclei of REF infected with the herpesvirus isolates from rodents contained great membrane vacuoles with a unit membrane, that had no morphological signs of invagination of the whole nuclear membrane; the vacuoles could have been derived from the inner sheet of the nuclear membrane. The vacuoles were localized mostly near the nuclear membranes, but neither fusion of the vacuolar membrane with, nor its derivation from the nuclear membranes was observed. Naked nucleocapsids of the herpesviruses formed in the nuclei were enveloped by membranes from the vacuoles and, in the course of gradual budding, they accumulated within these vacuoles. The vacuolar membrane at the site of nucleocapsid envelopment was somewhat thickened and electron optically more dense, as compared with other regions of the intranuclear vacuolar membrane (Fig. 3). In some sections, the intranuclear vacuole contained a smaller membrane vacuole, which contained unenveloped herpesvirus nucleocapsids. Invagination formed by the vacuole itself, while naked nucleocapsids could still be present in the nucleoplasm, could have been involved. However, this small vacuole did not contain the nucleoplasm alone (Fig. 4). Intranuclear vacuoles contained numerous enveloped herpesvirus particles with different types of nucleocapsids as illustrated in Fig. 1. Moreover, nucleocapsids with an electron translucent core and those with a ribbon-shaped core were observed (Figs 3 and 4). With isolates Nos 72 and 68, in the nuclei there occurred tubular structures of various lenght and a diameter of 33—35 nm. They occurred freely in the nucleoplasm close to the intranuclear granular structures and naked nucleocapsids (Fig. 5). Simultaneously, the nucleoplasm of these cells contained numerous bundles of fine fibrillar filaments 1.8-2.8 nm in diameter related to the naked nucleocapsids. The latter showed different types of cores as described above (Fig. 5). In addition to tubular structures situated freely in the nucleoplasm, the nuclei contained tubular structures related to the intranuclear membrane vacuoles (Fig. 6).

Ultrastructural changes in the cytoplasm of infected cells

The cytoplasm of REF infected with the five herpesvirus isolates was characterized by the formation of large inclusion bodies composed of electron-dense material with an extensive vacuolar system arranged in small aggre-

gates in different parts of the cytoplasm (Fig. 7) or in large aggregates at one place of the cytoplasm (Fig. 8). The accumulated, vacuoles contained numerous naked herpesvirus nucleocapsids located between vacuolar membranes. Enveloped virions occurred in the vacuoles rarely. Naked nucleocapsids with an electron translucent core, nucleocapsids enveloped with dense material, empty nucleocapsids or viral envelope membranes without nucleocapsids were more frequent in the vacuoles (Fig. 9).

The cytoplasm of cells infected with the isolate No. 78 contained large aggregates of electron-dense material, which surrounded numerous nucleocapsids with a densely stained electron-opaque core. At the periphery of dense bodies, usually smaller membrane vacuoles could be observed. At these membranes envelopment of naked nucleocapsids took place and frequently a part of dense material from the dense body was included into the envelope (Fig. 10).

Extracellular spaces contained numerous enveloped herpesvirus particles the nucleocapsids of which had a densely stained electron opaque core, while the envelope often contained a part of dense material from the dense intracytoplasmic bodies. Empty viral envelopes without nucleocapsids, containing only dense material from intracytoplasmic dense bodies, were frequent (Fig. 11). No budding of viral particles into the extracellular spaces was observed on the surface of cell membranes.

Discussion

Electron microscopy of REF at intervals after infection with five herpesvirus isolates from infection with five herpesvirus isolates from free living rodents showed that (*a*) all isolates induced in the host cells similar morphological changes, the formation of intranuclear tubular structures having been more marked isolates Nos 72 and 78 as was the formation of intracytoplasmic dense bodies in the isolate No. 78; (*b*) large membrane vacuoles in which envelopment of naked herpesvirus nucleocapsids synthesized in the nucleoplasm close to the aggregates of granular inclusions or diffuse granular inclusions were formed in the nuclei of infected cells; (*c*) naked nucleocapsids showed various types of core morphology — empty capsids or capsids with an electron-translucent core, capsids with a ribbon-shaped core, eventually capsids with a densely stained electron-opaque core; (*d*) extracellular viral particles contained in their envelopes also a part of dense material originating from intracellular cytoplasmic dense bodies, or there occurred viral envelope structures free of nucleocapsids but containing dense intracytoplasmic material; (*e*) the cytoplasm of infected cells contained either smaller vacuolar aggregates scattered in the vicinity of the nucleus, or large membraneous vacuoles containing numerous naked herpesvirus nucleocapsids which were enveloped into the vacuolar membrane and penetrated into the vacuoles by budding; (*f*) all infected cells moreover displayed characteristic features of developing herpesvirus infection of the cell — margination of nuclear chromatin, formation of lobate nuclei, disintegration of the nucleolus, absence

of nuclear membrane reduplication and no envelopment of naked nucleocapsids in the region of the nuclear membrane.

The studied herpesviruses isolated form small rodents induced mixed morphological changes in host cells, those which appear in cell cultures infected with freshly isolated human HSV (Nii, 1971b) as well as those occuring in cells infected with CMV (Middelkamp *et al.*, 1967); Fong *et al.*, 1980).

Nii (1971*b*) described envelopment of fresh HSV isolates (strains Iwa, Ko and Watanabe) by budding into perinuclear cisterns originating from the inner sheet of nuclear membrane as well as budding into cytoplasmic vacuoles from membranes of these vacuoles. Like in our experiments, no budding on the reduplicated nuclear membranes or on the cell surface membranes occurred in FL cells infected with freshly isolated human HSV (Nii, 1971*b*).

The appearance of non-enveloped nucleocapsids of HSV type 1 in the cytoplasm of infected cells is usually rare, but HSV type 2 strain G forms in the cytoplasm of HEp2 cells numerous non-enveloped or only partially enveloped nucleocapsids in early stages of infection. The site of envelopment may depend on the MOI at a cetain time and stage of infection. Nucleocapsids were also observed in the cytoplasm of cells infected with HSV type 1, but the time of their appearance varied in different experimental systems (Nii, 1971*b*).

Infection of cells with guinea-pig cytomegalovirus (GP-CMV) and human CMV induces ultrastrustural changes characterized by intranuclear inclusions containing clumps of electron-dense fibrillar structures with a diameter of 10 nm and viral nucleocapsids at different stages of development. Nearly all CMV nucleocapsids are enveloped on the inner sheet of the nuclear membrane and accumulate as enveloped particles in cytoplasmic membrane--bounded vacuoles originating from the outer sheet of the nuclear membrane (Fong *et al.*, 1980). Homogeneous electron-dense material forming inclusion bodies in the cytoplasm of cells infected with human CMV together with the appearance of similar structures in the nuclei of infected cells suggests that its formation might result from an overproduction of some herpesviral structural proteins (Ruebner *et al.*, 1965; Smith and DeHarven, 1973; Iwasaki *el al.*, 1973).

Tubular structures with a diameter of 40—60 nm were present in the nuclei of primary guinea pig embryo cell cultures infected with GP-CMV; in the nuclei of rabbit kidney cells infected with Herpesvirus cuniculi two different tubular structures could be detected: smaller ones with a diameter of 65 nm and larger ones with a diameter of 90 nm (Middelkamp *et al.*, 1967; Nii and Yasuda, 1975). Unusual tubular structures with a diameter of 70—80 nm appeared in cells infected with HSV type 2 and grown in medium containing 2-deoxy-D-glucose (Iwasaka *et al.*, 1978). The origin and function of these tubular structures during the replication cycle of herpesviruses remains obscure.

The morphology of REF infected with five herpesvirus isolates from small rodents revealed ultrastructural changes characteristic of all herpesviruses

studied on the one hand. On the other, some of the isolates induced changes resembling those induced by freshly isolated human HSV in FL cells (Nii, 1971b). The formation of tubular structures in the nuclei of infected cells is more frequent with cytomegaloviruses, but the process of envelopment of nucleocapsids in cells infected with the isolates from small rodents differed from the process of envelopment in human CMV or GP-CMV and was almost identical with that observed in FL cells infected with freshly isolated human HSV (Nii, 1971*b*).

The formation in the cytoplasm of REF of multivacouolar membrane structures containing unenveloped nucleocapsids is considered unique. Unenveloped nucleocapsids occur more frequently in the cytoplasm of cells infected with HSV, while CMV in the cytoplasm always is enveloped. The formation in the cytoplasm of large electron-dense inclusion bodies containing numerous unenveloped nucleocapsids, observed in REF infected with some herpesvirus isolates from small rodents, has so far not been detected in cells infected, with either HSV or CMV. In general, the ultrastructural development of the rodent isolates in REF seems to resemble that of freshly isolated human HSV in cell cultures more than the changes induced in cells by CMV or laboratory-maintained strains of HSV.

Acknowledgements. The outstanding technical assistance in specimen preparation for electron microscopy and photodocumentation of Mr. B. Holec and Mr. N. Dokoupil is greatly appreciated.

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Explanation of Electron Micrographs (Plates XIII-XVIII):

Fig. 1. Nucleus of REF 7 days p. i. with the herpesvirus isolate No. 76. Aggregates of granular inclusions (G) close to the nuclear membrane are connected with viral nucleocapsids. N — nucleus. × 20 000.

Fig. 2. Diffuse granular inclusion (G) in the nucleus of REF 5 days p. i. with the herpesvirus isolate No. 60 is connected with the formation of virus nucleocapsids. × 99 000.

Fig. 3. Nucleus of REF 7 days p. i. with herpesvirus isolate No. 72. Within the nucleus, a membranous vacuole with enveloping nucleocapsids (arrows) budding inside of the vacuole. N — nucleus. × 82 500.

Fig. 4. Nucleus (N) of REF 7 days p. i. with herpesvirus isolate No. 78 contains a membranous vacuole with enveloped virions. Small vacuole (astrerisk) contains naked nucleocapsids. × 99 000.

Fig. 5. Nucleus (N) of REF 7 days p. i. with herpesvirus isolate No. 68. Within the nucleus, tubular structures with a diameter of 33–35 nm (arrow) and bundles of fine fibrillar structures (F) with a diameter of 1.8–2.8 nm, connected with nucleocapsid formation. × 69 300.

Fig. 6. Nucleus (N) of REF 7 days p. i. with herpesvirus isolate No. 72. Numerous tubular structures with a diameter of 33–35 nm (arrow) located in the region of nuclear membrane vacuole. × 69 300.

Fig. 7. Lobate nucleus (N) of REF 7 days p. i. with herpesvirus isolate No. 68. Accumulation of small membrane vacuoles (asterisks) in the cytoplasm close to the nuclear membrane. × 21 000.

Fig. 8. Large aggregate of membrane vacuoles and electron dense material in the cytoplasm of REF 5 days p. i. with herpesvirus isolate No. 72 × 26 250.

Fig. 9. Accumulation of membrane vacuoles with numerous naked nucleocapsids in the cytoplasm of REF 7 days p. i. with herpesvirus isolate No. 60. × 56 700.

Fig. 10. Electron-dense body with dense material and with numerous naked nucleocapsids in dense cytoplasmic material of REF 7 days p. i. with herpesvirus isolate No. 78. × 79 200.

Fig. 11. Extracellular enveloped herpesvirus particles in REF 5 days p. i. with herpesvirus isolate No. 78. Electron-dense material included into the envelope of viral particles; viral envelope structures without nucleocapsids partialy filled with dense material from intracytoplasmic dense inclusions. × 79 200.