Intervirology

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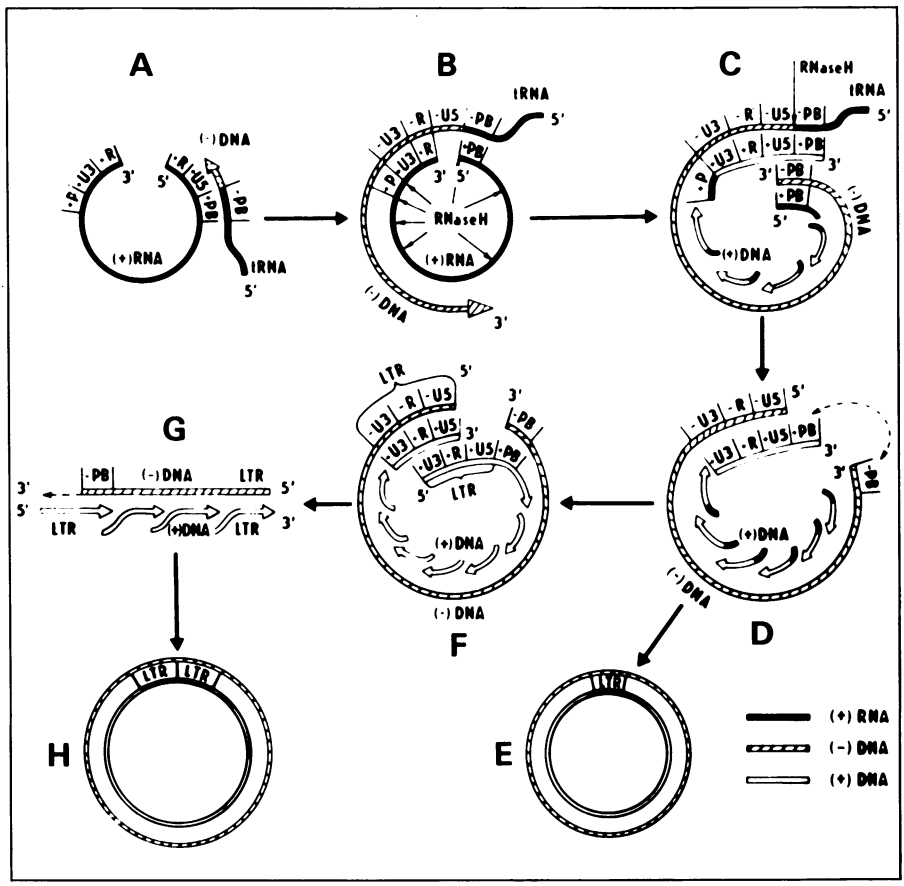

Steps in reverse transcription of viral genomic RNA to proviral DNA, slightly modified from Junghans et al. Svoboda (page 17).

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Vol. 26, 1986

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°	degree centigrade (Fahrenheit will not be used)
A, C, G, T, U	adenine, cytosine, guanine, thymine, uracil
AMP	5′-monophosphates of adenosine
CMP	– cytidine
GMP	– guanosine
TMP	– thymidine
UMP	– uridine
ADP, etc.	adenosine 5′-diphosphate, etc.
ATP, etc.	adenosine 5′-triphosphate, etc.
CPE	cytopathic effect
DEAE-cellulose	diethylaminoethylcellulose
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EDTA	ethylenediaminetetra-acetate
EOP	efficiency of plating
g	acceleration of gravity
ID_{50}	median infective dose
LD_{50}	median lethal dose
M	molar
MEM	minimum essential medium
MOI	multiplicity of infection
N	normal
ng	nanogram
nm	nanometer (10^{-9} m)
PAGE	polyacrylamide gel electrophoresis
PFU	plaque forming units
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
SD	standard deviation
SDS	sodium dodecyl sulfate
sp gr	specific gravity
TCD_{50}	median tissue culture infective dose
Tris	tris (hydroxymethyl) aminomethane (2-amino-2-hydroxymethylpropane-1,3-diol)
UV	ultraviolet
V	volt
W	watt

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Studies on the Morphogenesis of Murine Cytomegalovirus

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Federal Research Centre for Virus Diseases of Animals, Tübingen, FRG

Key Words. Murine cytomegalovirus · Morphogenesis · Electron microscopy · Multicapsid virions

Summary. Two modes of assembly of murine cytomegalovirus (MCMV) were observed in cultured mouse embryo fibroblasts, generating two morphologically different types of viral particles: monocapsid virions and multicapsid virions. The assembly of nucleocapsids appeared to be the same for both types of morphogenesis. Three successive stages of intranuclear capsid formation could be distinguished: capsids with electron-lucent cores, coreless capsids, and capsids with dense cores. Some of the capsids were enveloped at the inner nuclear membrane to form monocapsid virions, which were first detectable in the perinuclear cisterna. Other capsids left the nucleus via nuclear pores and usually entered cytoplasmic capsid aggregates that received an envelope by budding into extended cytoplasmic vacuoles, thereby forming multicapsid virions. Since the formation of multicapsid virions is not restricted to cell culture conditions and also occurs in vivo in immunosuppressed mice, multicapsid virions may play a role in the pathogenesis of cytomegalovirus infection.

Cytomegaloviruses cause severe clinical symptoms only in the immunologically immature or immunosuppressed host [for review see Hamilton, 1982], indicating that in the immunocompetent host the immune system is involved in the control of acute infection and the establishment of viral latency. Infection of mice with murine cytomegalovirus (MCMV) proved to be a convenient virus-host model to study aspects of herpesvirus latency, recurrence, and pathogenesis [Brody and Craighead, 1974; Jordan et al., 1977; Mayo et al., 1977; Brautigam et al., 1979; Shanley et al., 1982]. A morphologic feature special to MCMV is the preferential formation of multicapsid virions in addition to the regular development of monocapsid virions during in vitro infection of embryonic fibroblasts [Hudson et al., 1976a], whereas the generation of twin capsid forms or oligocapsid virions is a rare event in cells infected with herpes simplex virus [Nii et al., 1968; Watson, 1973] and also in cells infected with

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rodent cytomegaloviruses other than MCMV [Fong et al., 1979; Bruggeman et al., 1982]. Multicapsid virions of MCMV were not observed in salivary gland tissue during infection of immunocompetent mice. Only recently has it been observed that multicapsid virions are also formed in vivo in fibroblastic lung cells during infection of immunosuppressed mice [Reddehase et al., 1985]. The origin of the virion envelope may be decisive for its molecular composition and thus for its immunogenic properties. Therefore, we studied the ultrastructural morphogenesis of both types of MCMV virions, monocapsid as well as multicapsid virions.

Materials and Methods

Virus and Infection Conditions

MCMV (strain Smith, ATCC VR-194) was obtained from the American Type Culture Collection, Rockwille, Md., USA and was propagated in BALB/c mouse embryo fibroblast cells. Tertiary fibroblasts were prepared from 15- to 17-day-old BALB/c embryos as described [Keil et al., 1984]. Cells were infected with MCMV by employing the technique of centrifugal (800 *g* for 30 min) enhancement of infectivity [Osborn and Walker, 1968; Hudson et al., 1976b], which resulted in an MOI of 20 PFU per cell. To inhibit viral DNA synthesis, cells were infected with MCMV in the presence of 250 μg/ml phosphonoacetic acid or 20 m*M* hydroxyurea (HU) and maintained in the presence of the inhibitor.

Electron Microscopy

For electron microscopy, in situ fixation of cell monolayers (16–28 h postinfection) was performed with 2.5% glutaraldehyde in 0.1 *M* sodium cacodylate buffer (pH 7.2) for 2 h. After rinsing with cacodylate buffer, the cells were gently scraped off, collected by centrifugation, and postfixed in 1% osmium tetroxide. Specimens were dehydrated in acetone and embedded in Araldite. Sections were cut with a Reichert OmU3 ultramicrotome. Semithin sections (1 μm) were stained with toluidine blue to select sites of interest by light microscopy. Ultrathin sections of selected sites were then stained with uranyl acetate and lead citrate, and examined in a Siemens 101 electron microscope at 80 kV.

Results

Assembly of Nucleocapsids

The technique of centrifugal enhancement of infectivity [Osborn and Walker, 1968; Hudson et al., 1976b] was applied to synchronize the inoculation. Two hours after infection, more than 90% of the mouse embryo fibroblasts in a semiconfluent monolayer expressed the first gene product of MCMV, the immediate-early phosphoprotein IE pp89 [Keil et al., 1985] in the nucleus, as detected by indirect immunofluorescence (not shown). This finding proved that viral replication started with a high degree of synchronicity. Viral DNA synthesis defining the beginning of the late phase of viral replication occurs at about 16 h postinfection [Ebeling et al., 1983], and at that time ultrastructural alterations could be detected by electron microscopy.

Fibroblasts displayed enlarged nuclei with a pale nucleoplasm and delicately marginated chromatin. Faint amorphous material was arranged in two or three focal areas in the nuclear matrix between the nucleoli. Coinciding with increasing staining affinity, the amorphous material acquired a more reticular appearance, and the first forms of viral capsids, typified by an electron-lucent core, emerged in the nucleus. Thereupon the focal areas fused to form a single large inclusion body composed of diffusely or reticularly arranged amorphous material. This inclusion occupied the whole center of the nucleus and was surrounded by a clear zone of nucleoplasm that separated the inclusion from the

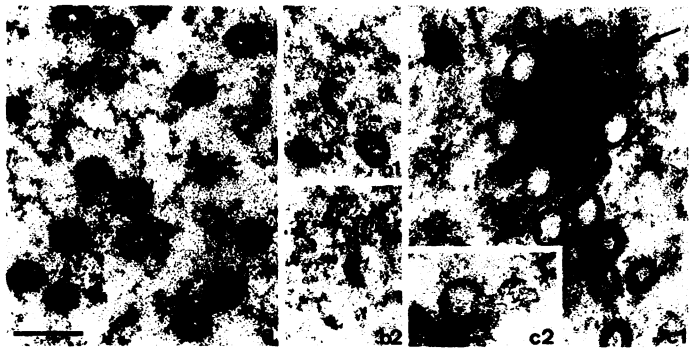

Fig. 1. Capsids in the nucleus of an MCMV-infected cell. **a** Capsids with electron-lucent core. **b1,2** Incompletely formed capsids of the type as shown in **a. c1** Coreless capsids; arrow: nucleocapsid. **c2** Empty capsid possessing a distinct orifice. Bar marker represents 200 nm.

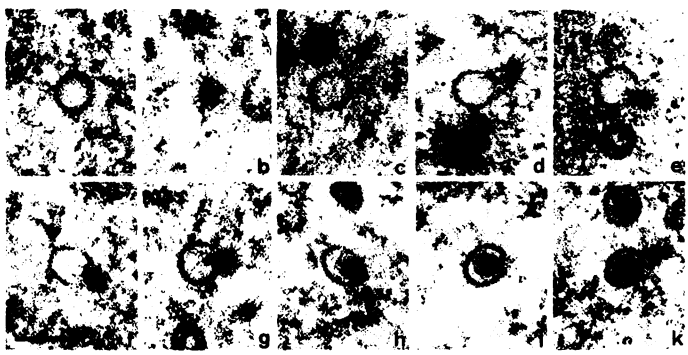

Fig. 2. Different stages in intranuclear incorporation of spider-like filaments (deoxyribonucleoprotein strands) into coreless capsids finally forming the mature nucleocapsid. **a** Coreless capsid with an orifice. **b** Spider-like filamentous structure located in the nuclear matrix. **c** Contact between an osmiophilic 'spider' and an empty capsid. **d–i** Different forms of association of 'spiders' with empty capsids. **k** Mature nucleocapsid. Bar marker represents 200 nm.

nuclear membrane. This zone was identical to a halo observed by light microscopy. Frequently, single capsids or clusters of capsids were interspersed among the amorphous material of the inclusion body. Only in single cases were the majority of capsids located in the nucleoplasm of the halo region (data not shown).

At that stage three types of capsids were observed simultaneously in the nucleus of individual MCMV-infected cells: (i) Capsids with a diameter of 100 nm containing an electron-lucent core of hexagonal symmetry (fig. 1a). Incompletely formed crescent shaped capsids with crescent shaped cores were encountered occasionally (fig. 1b1,2). These putatively represented capsids in a stage of assembly. (ii) Capsids with a hexagonal profile devoid of a core (fig. 1c1). These capsids differed in size but usually were slightly larger than those described above. (iii) Capsids containing an electron-dense fuzzy or more condensed core that was located in the center (fig. 1c1, arrow; fig. 2k). Their diameters were 100 nm. These capsids were designated nucleocapsids and were considered to represent mature capsids containing viral DNA.

As already mentioned above, the first capsids emerging in the nucleus were those with

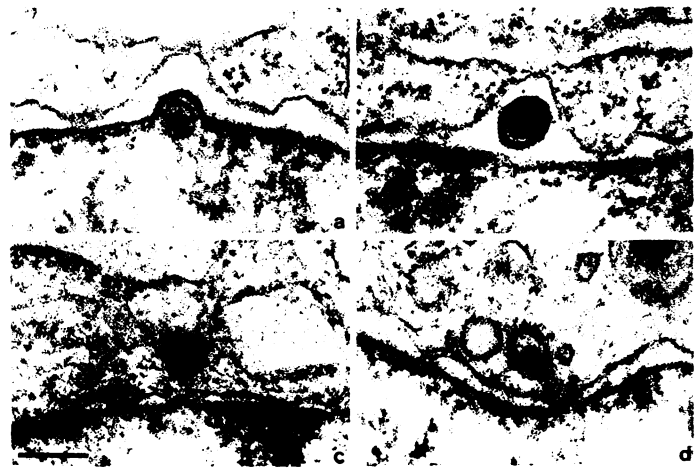

Fig. 3. Nucleocapsids leaving the nucleus. **a** Budding of a nucleocapsid into the perinuclear space. **b** Enveloped monocapsid virion freely lying in the perinuclear cisterna. **c, d** Nucleocapsids located in the cytoplasm close to nuclear pores. Bar marker represents 200 nm.

electron-lucent cores. The formation of nucleocapsids was selectively halted at this stage when cells were infected in presence of HU, whereas any capsid assembly was prevented when viral DNA synthesis was blocked with phosphonoacetic acid. Coreless capsids were formed only in the absence of HU when transition to the late (γ) phase of viral gene expression was not inhibited. These findings strongly suggest that coreless capsids represent a more advanced stage in the assembly of nucleocapsids, but since intermediate stages were not observed it could not be established whether capsids with electron-lucent cores represented direct predecessors of coreless capsids or whether they were an independent structure of as yet unknown function.

Coreless capsids possessed a distinct orifice (fig. 1c2, 2a). Electron-dense filamentous material with a spider-like appearance (most likely representing deoxyribonucleoprotein strands) originated separately in the nuclear matrix (fig. 2b). These osmiophilic 'spiders' occasionally had fibers protruding towards the empty capsids (fig. 2c). Often they were detected in different stages of association with coreless capsids (fig. 2d–i). Capsids with electron-lucent cores devoid of DNA also accumulated in the presence of HU in cells infected with herpes simplex virus and, after removal of the inhibitor, EDTA-sensitive nucleoprotein filaments have been observed in structural association with capsids, which appear to be stages in a process of entering [Friedmann et al., 1975]. It is plausible that the spider-like filaments of MCMV enter rather than leave the coreless capsids, finally forming the electron-dense compact deoxyribonucleoprotein core that is characteristic of the mature nucleocapsid (fig. 2k). In conclusion, apart from minor differences in ultrastructural details, the assembly of MCMV nucleocapsids follows the scheme common to herpesviruses.

Formation of Monocapsid and Multicapsid Virions

Nucleocapsids were detected in intimate contact with the inner nuclear membrane (some of them during the process of budding) and were partly enveloped by a thickened

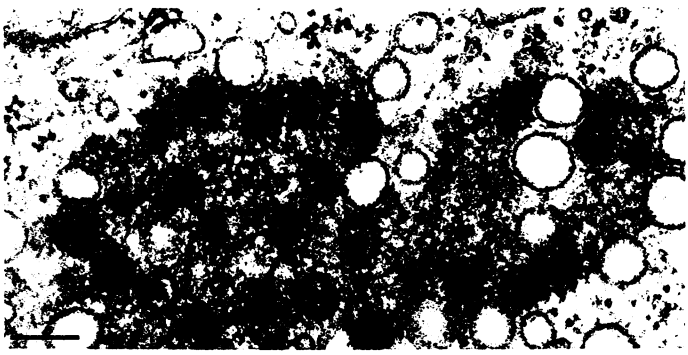

Fig. 4. Cytoplasmic nucleocapsids arranged in clusters and embedded in fine granular material. Bar marker represents 200 nm.

area of the inner nuclear membrane (fig. 3a). The budding process resulted in the release of enveloped monocapsid virions into the perinuclear cisterna (fig. 3b).

In the cytoplasm, nonenveloped single nucleocapsids were not randomly distributed, but appeared in conspicuous proximity to nuclear pores (fig. 3c, d), strongly suggesting that these nucleocapsids had just left the nucleus via that passageway. Passing through may be a rapid process, which would explain why nucleocapsids were not observed in the pore. The antithesis predicts de-envelopment of monocapsid virions [Severi et al., 1979] followed by penetration into the nucleus via the nuclear pores. Such a view, however, is neither plausible nor has any precedent in published reports on herpesvirus morphogenesis.

Nucleocapsids aggregated in the cytoplasm and were embedded in electron-dense deposits, consisting of an amorphous, fine granular substance (fig. 4). At lower magnification such clusters appeared as compact cytoplasmic inclusions. Frequently, the nucleocapsids were arranged predominantly at the margin of the inclusion. Only rarely were the amorphous matrix material and nucleocapsids seen separately in the cytoplasm. Besides mature nucleocapsids, coreless capsids also entered the clusters, whereas capsids containing the electron-lucent altered form of the core described above (fig. 1a) were never encountered outside the nucleus.

In the Golgi region, clusters consisting of variable numbers of embedded capsids (occasionally only one) were enveloped by budding into cytoplasmic vacuoles (fig. 5a, b), thereby forming either multicapsid virions or monocapsid virions that contained a significant amount of matrix material as tegument, in contrast to those monocapsid virions that were enveloped at the inner nuclear membrane. Multicapsid virions as well as monocapsid virions formed by either mechanism were enclosed in smooth cytoplasmic vacuoles (fig. 5c, d) and released into the extracellular space (fig. 5e). Studies on virion morphogenesis in various tissues of virus-infected immunosuppressed mice have shown that in the absence of immune control, MCMV replicates in vivo exactly as demonstrated for cultured fibroblasts. The presence of monocapsid virions in the perinuclear cisterna and the release of multicapsid virions have been shown for infected fibroblastic

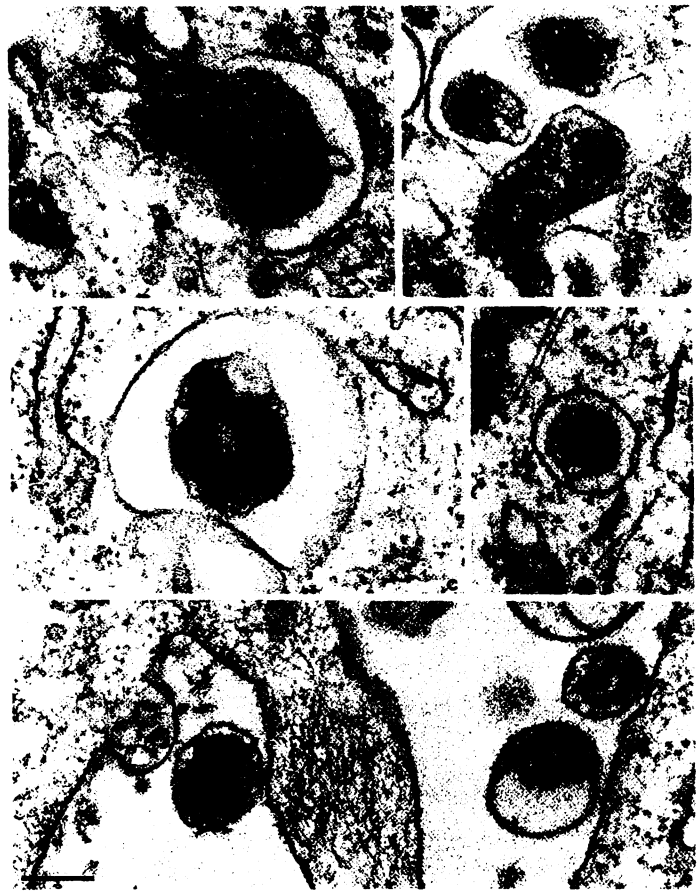

Fig. 5. Envelopment of cytoplasmic nucleocapsids. Simultaneous budding of nucleocapsids into cytoplasmic vacuoles thereby forming multicapsid virions containing various numbers of nucleocapsids **a, b**. Multicapsid **c** and monocapsid **d** virions enclosed in cytoplasmic vacuoles and released into the extracellular space **e**. Bar marker represents 200 nm.

cells of the alveolar septa during interstitial MCMV pneumonia [Reddehase et al., 1985]. From our findings, we envision the course of MCMV morphogenesis as schematically outlined in fig. 6.

Discussion

MCMV differs from other members of the herpesvirus family by producing two different types of viral particles simultaneously in the same individual cell: monocapsid virions and multicapsid virions. This uncommon feature may account for properties important for the induction of an immune response and for the course of pathogenesis. To clarify the origin of the virion envelopes we studied the ultrastructural morphogenesis of MCMV.

It was found that both types of virion have the first steps of morphogenesis in common – the assembly of nucleocapsids in the nuclei of infected cells. In accordance with findings of Lussier et al. [1974] and Kurimura et al. [1977], three types of capsids were observed: capsids with electron-lucent cores, coreless capsids, and capsids with dense cores. Consistent with the assembly of herpes simplex

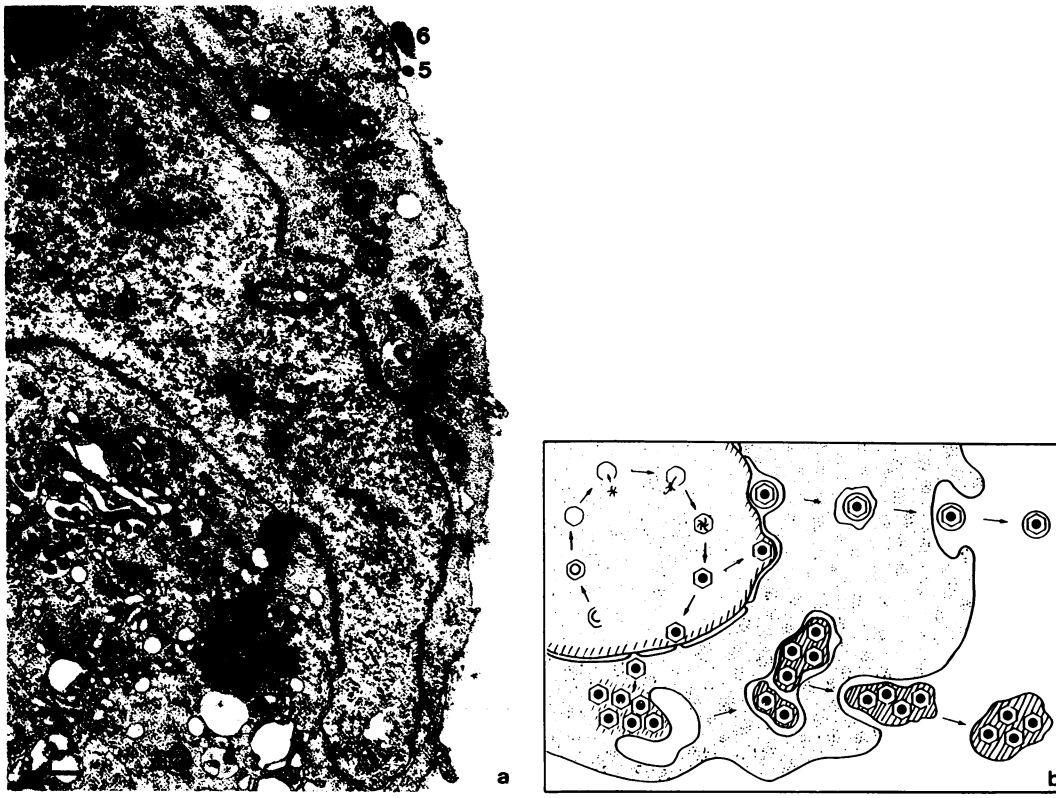

Fig. 6. a Part of an MCMV-infected cell. (1) Intranuclear capsids in different stages of assembly. (2) Accumulation of cytoplasmic capsids. (3) Budding of cytoplasmic capsids. (4) Intravacuolar multicapsid virion. (5) Extracellular monocapsid virion. (6) Extracellular multicapsid virion. Bar marker represents 1 μm. **b** Schematic representation of the supposed course of MCMV morphogenesis.

virus capsids [Schwartz and Roizman, 1969], capsids with electron-lucent cores were never seen as free particles or enveloped virion-like particles outside the nucleus, whereas for the infection of human fibroblasts with strains of human cytomegalovirus (HCMV), Irmiere and Gibson [1983] have reported noninfectious enveloped particles (NIEPs) which are characterized by capsids containing a core similar in structure to that of intranuclear HCMV B-capsids, which correspond to the MCMV capsids with electron-lucent cores. In a more recent report the same authors provide evidence that NIEPs are in fact enveloped B-capsids and suggest that an assembly protein forming the core before DNA packaging is modified or removed in conjunction with DNA packaging [Irmiere and Gibson, 1985]. Our studies with HU and results obtained for herpes simplex virus by Nii et al. [1968] and Friedmann et al. [1975] allow the analogous conclusion, namely that capsids with electron-lucent cores do not contain the viral genome and represent a premature

stage in nucleocapsid assembly. Likewise it is now established for cytomegaloviruses [Kanich and Craighead, 1972; Haguenau and Michelson-Fiske, 1975; Kurimura et al., 1977] and for herpes simplex virus [Friedmann et al., 1975; Luetzeler and Heine, 1978] that viral deoxyribonucleoprotein strands enter the coreless capsids to form mature nucleocapsids.

Budding at the inner nuclear membrane is the most frequent way of monocapsid virion formation for most herpesviruses [Shipkey et al., 1967; Darlington and Moss, 1968]. Cytomegaloviruses represent an exception to the rule since virions receive the envelope predominantly by budding into cytoplasmic vesicles [Todd and Storz, 1983]. Another characteristic of cytomegalovirus infections is the formation of cytoplasmic inclusions consisting of embedded nucleocapsids [Luse and Smith, 1958; Ruebner et al., 1966; Berezesky et al., 1971; Craighead et al., 1972; Hudson et al., 1976a; Fong et al., 1979; Storz et al., 1984]. Release of enveloped nucleocapsid aggregates containing 1 to more than 20 nucleocapsids, i.e. multicapsid virions, into the extracellular space is, however, a prominent feature only during MCMV infection [Hudson et al., 1976a]. The occasional detection of bicapsid virions in other rodent cytomegalovirus infections [Fong et al., 1979; Bruggeman et al., 1982] can be explained by incidental budding of two nucleocapsids into the same vesicle.

The site of envelopment of MCMV nucleocapsid aggregates remained to be elucidated. Because it was considered unlikely that extended aggregates bud at the nuclear membrane or into cytoplasmic vesicles in the way single nucleocapsids do [Leestma et al., 1969], budding at the cell membrane appeared to be a plausible alternative. Nonetheless, our studies have now established that the aggregates do not bud at the cell membrane, but bud exclusively into extended cytoplasmic vacuoles derived from the Golgi apparatus. On the other hand, monocapsid virions developed only incidentally by this mode from nucleocapsids that did not join aggregates. MCMV monocapsid virions were primarily generated by budding at the inner nuclear membrane. All three types of MCMV virions were transported through the cytoplasm enclosed by cytoplasmic unit membranes and were released into the extracellular space by exocytosis. In conclusion, neither multicapsid virions nor the two types of monocapsid virions received the envelope by budding at the cell membrane, and the origin of the multicapsid virion envelope differed from that of most monocapsid virions.

These results could have an influence on the interpretation of recent findings. First, production of multicapsid virions in fibroblast cells in culture and of monocapsid virions in vivo in cells of the salivary glands has been related to attenuation and virulence, respectively [Osborn and Walker, 1970; Chong and Mims, 1981; Jordan and Takagi, 1983]. Since monocapsid virions and multicapsid virions gain their envelope at different sites it is possible that they differ in the composition of their envelopes and hence also in their properties. Second, none of the three types of virions is enveloped at the cell membrane. It is therefore conceivable that antigens which are derived from the virion envelope after integration into the cell membrane from without during penetration of the virion are not necessarily presented at the cell membrane in an antigenic form during intracellular virion assembly. This might explain why a cytolytic T-lymphocyte clone specific for vi-

rion structural antigen, clone S1 [Reddehase et al., 1986a], did not detect this antigen in the late phase of infection at a time when high amounts of virus were produced and released [Reddehase et al., 1986b].

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