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Herpes Simplex Virus and Human Cytomegalovirus Replication in WI-38 Cells

I. Sequence of Viral Replication

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A comparison, under standardized conditions, of herpes simplex virus (HSV) and human cytomegalovirus (CMV) revealed differences in viral morphology, in the timing of their infectious cycles, and in several morphological events during those cycles. Structural distinctions between the two viruses included the coating of unenveloped cytoplasmic CMV capsids, but not those of HSV, and a variation in the structure of their cores. Since the two cycles were carried out in the same host cell strain under conditions of one-step growth (input multiplicity = 10 PFU/cell), it was possible to construct time scales locating the major events of each cycle. Comparison of the two showed that HSV replicated and released progeny within 8 h postinfection, whereas CMV required 4 days. These results correlated well with those of concurrent plaque assays. Within the longer CMV cycle, most of the major events appeared retarded to a similar degree, and no obvious limiting step in particle production could be identified. Distinctions between the two cycles included the following: condensation of the chromatin in HSV- but not CMV-infected cells; the greater tendency of HSV to produce membrane alterations; and the appearance of cytoplasmic dense bodies in CMV- but not HSV-infected cells. Identification of these differences even under identical conditions of culture and infection strongly implies that they result from intrinsic differences in the nature of the viruses, and are not caused by variations in experimental conditions.

The members of the herpesvirus group share common features including a similar morphology, a nuclear site of capsid assembly, and similar modes of maturation and release, yet differ significantly in other parameters such as the length of the replication cycle, the host range, and the yield of infectious cell-free virus (12, 17, 32). To date many of the studies of individual herpesviruses have been carried out under different conditions in different host cell systems, making it difficult to determine whether observed dissimilarities were due to the intrinsic nature of the viruses or to the variations in the systems employed. We report here on a comparison of the replicative cycles of two herpesviruses carried out under standardized conditions in the same host cell system, using equivalent multiplicities of infection. To establish a time scale for each cycle, a multiplicity sufficient to produce a synchronous one-step growth cycle was used.

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MATERIALS AND METHODS

Cells. WI-38 cells were obtained from the American Type Culture Collection in the 16th passage. They were grown as stationary monolayers in minimal essential medium (MEM) with Earle's salts plus 10% fetal calf serum (FCS), 7.7 mM $NaHCO_3$, 100 μg of streptomycin per ml, and 100 U of potassium penicillin G per ml. Tests for mycoplasma contamination were negative.

Virus stocks. Herpes simplex virus (HSV), strain HF (8) was received from the American Type Culture Collection, as was human cytomegalovirus (CMV), strain AD-169 (28). Both were free of mycoplasma. Virus-infected cells were maintained on MEM with 2% agamma calf serum (MEM 2), and were harvested when 85 to 90% showed cytopathic effects. Mechanical homogenization was followed by centrifugation at 500 $\times$ g for 10 min and filtration through prewashed Millipore filters with pore size of 0.8 and then 0.45 μm (6). Clarified suspensions of HSV were frozen immedi-

ately, whereas those of CMV were concentrated 10- to 20-fold by negative pressure ultrafiltration through dialysis tubing (25). The frozen stocks were titered by plaque assay.

Plaque assay. Cell-free virus was assayed by the procedures of Wentworth and French for both HSV (29) and CMV (30), except that CMV was diluted in medium lacking bicarbonate, and fixed monolayers were stained with 1% aqueous methylene blue.

Infection of cell monolayers. Preconfluent monolayers were infected with 0.1 ml/plate of cellfree virus at a multiplicity of 10 PFU/cell. After adsorption for 90 min at 37 C and 5% CO_2, the cultures were washed once and incubated in 2 ml of fresh MEM 2. HSV-infected samples were taken at 0, 2, 3, 4, 5, 6, 8, 10, 12, and 24 h postinfection, with uninfected controls at 0 and 24 h. A sample of medium from each plate was frozen and subsequently titered for released virus. CMV-infected cells were fixed at 12-h intervals over a period of 8 days, with uninfected controls at 0 and 7.5 days. Duplicate plates were taken at each sampling time, one for titration of released and cell-associated virus and the other for electron microscopy.

Electron microscopy. The monolayers were fixed and embedded in situ by a modification of the method of Brinkley et al. (3). Sections were stained with uranyl acetate and lead citrate, coated with carbon, and examined in a modified Siemens 1A electron microscope. The main modification was a shorter objective lens focal distance (H. Armbruster, Siemens Inc., Iselin, N.J.).

For negative staining, unfixed viral specimens were adsorbed to grids covered by thin carbon films, and then stained with 2% aqueous phosphotungstic acid (PTA), pH 4.8.

RESULTS

Virus growth curves. The growth curves for the cycles studied by electron microscopy (Fig. 1) indicate that, as expected with an input multiplicity of 10 PFU/cell, conditions of one-step infection were achieved with both viruses. This fact was independently confirmed by infectious center assays.

Viral morphology. The major structural differences between the two viruses were as follows: (i) In negatively stained samples, the cores of HSV appeared to be continuous structures with a wall of uniform thickness (Fig. 2d), whereas those of CMV resembled aggregates of globular subunits (Fig. 2b). This distinction in core structure was also evident in thin sections, where CMV but not HSV cores had a beaded appearance in cross section (Fig. 3c, e). Despite a recent report on herpes simplex morphology (9), we were unable to find any evidence indicating a toroidal structure for the core. (ii) Unenveloped CMV capsids located free in the cytoplasm were coated by a layer of fibrillar material applied directly to the exterior of the capsid (Fig. 3d), whereas free cytoplasmic capsids of HSV were never coated (Fig. 3f). Coats were never present on those capsids of HSV or CMV located within intact nuclei (Fig. 3e). Other features of viral morphology, including the variety of structural types usually observed with herpesviruses (25), are illustrated in Fig. 2 and 3.

Comparison of paired HSV and CMV means, using Student's t test, showed no significant differences between any dimensions of the two ($\alpha = 0.1$). In thin section, enveloped particles averaged 174.0 nm in diameter, capsids averaged 106.4 nm, and cores averaged 64.3 nm. The average thickness of the coat on unenveloped cytoplasmic CMV capsids was 24.4 nm.

CMV replication cycle. By electron microscopy the first visible sign of infection was the rounding up of individual uninucleate cells between 12 and 24 h postinfection. The reniform nuclei were displaced to the periphery of the cytoplasm, while a prominent Golgi apparatus (Fig. 4) occupied the center of the cell.

Less frequently cells fused to produce small polykaryocytes usually containing 3 to 4 nuclei. These were often arranged in a ring surrounding the Golgi elements.

By day 3 capsids were assembling in the nucleus. At this stage they were all of one morphological type, namely those containing "empty" cores. Occasionally a capsid and core appeared to be developing in synchrony. Free cores without a surrounding capsid were not observed. Capsids with dense cores began to appear between 3 and 3.5 days.

At 3.5 days most of the capsids were closely associated with a distinctive skein-like nuclear inclusion (Fig. 5), although smaller numbers were found in the clear zone of nucleoplasm which characteristically separated these inclusions from the nuclear membrane.

Envelopment at the nuclear membrane began between 3 and 3.5 days. Capsids of all morphological types budded through the inner nuclear membrane, either directly into the perinuclear cisterna (Fig. 6), or into sac-like membranous invaginations commonly found in the nucleoplasm (Fig. 7). Rupture of the nuclear membrane was not observed at these early stages.

In the period between 2 and 3 days postinfection, there was a transient increase in the number of 55- to 60-nm intranuclear granules (Fig. 8, 9). Although previously reported by others (23) and interpreted as possible viral nucleoids, these seem more closely to resemble the perichromatin granules of normal cells, both

in size and in the presence of a clear area or "halo" separating many of them from heterochromatin.

From the perinuclear cisterna, enveloped particles usually moved into the cytoplasm within smooth vacuoles, formed by the evagination of the outer nuclear membrane. Nonlytic release of viruses within vacuoles occurred when the vacuolar membrane fused with the plasmalemma, thereby extruding the enveloped particle. Alternatively, particles could leave the perinuclear cisterna via the channels of the rough endoplasmic reticulum (Fig. 10), and could travel through these channels for variable distances. Since no continuities were ever found between the rough endoplasmic reticulum and the plasmalemma, the viruses within the reticulum must exit from the cell by a discontinuous route involving intermediate vacuoles.

The effect of CMV on cellular membranes was minimal. The nuclear membrane reduplication which is characteristic of herpesviruses occurred only to a limited extent (Fig. 11). Reduplication seldom involved more than one extra membrane pair or more than a short section of the nuclear membrane. Similarly, cell fusion was not common, and those polykaryocytes observed rarely had more than 3 or 4 nuclei. In the cytoplasm there was a proliferation of Golgi vesicles and stacked saccules (Fig. 12), but these retained their normal morphologies.

Within the nuclei there was no noticeable condensation or margination of the chromatin either at this or later stages of the infection.

Despite the apparently intact nuclear membranes, unenveloped capsids were routinely observed in the cytoplasm beginning at 4 days. These capsids, all of which were coated, were most numerous in the immediate vicinity of the nucleus and in the Golgi region (Fig. 12e). In the Golgi zone many budded into a variety of cytoplasmic vacuoles including multivesicular bodies, phagolysome-like vacuoles, and a characteristic small, clear vacuole with a crescent-shaped cross section (Figs. 12a–c). As a result of this process the capsids simultaneously acquired an envelope and became enclosed in a surrounding vacuole, making them virtually identical to viruses enveloped at the nuclear membrane.

Concurrent with capsid assembly and envelopment, a homogenous electron-dense material began to accumulate in the cytoplasm, especially in the Golgi region (Fig. 12e). It appeared first in small, scattered foci which lacked a limiting membrane. These retained

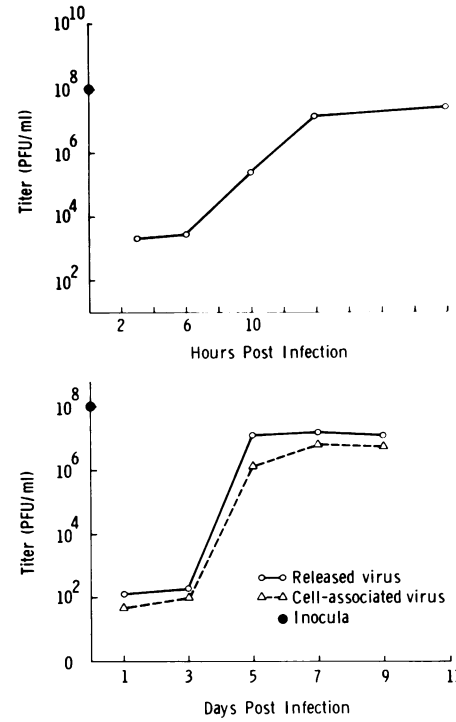


FIG. 1. *One-step growth curves showing the titers of released virus for HSV (upper curve), and both released and cell-associated virus for CMV (lower curve).*

regular spherical outlines while gradually increasing in size. By day 5 many foci were acquiring an envelope by budding into the same types of vacuoles as CMV capsids (Fig. 12d, e). Individual vacuoles often contained a mixed population of both these enveloped dense bodies and enveloped CMV. The dense bodies were released from the cell in the same manner as virus.

In later stages, a variety of distinctive nuclear inclusions were observed (Fig. 13, 14). These may represent sequential stages in a general compaction or condensation of the skein-like inclusions described above.

Cell lysis commonly occurred at 7 to 8 days postinfection.

HSV replication cycle. The most obvious dissimilarity between the cycles of HSV and CMV was in the length of time necessary for replication (Fig. 1). A comparison of the timing of the major events in the two cycles is shown in Table 1.

Although the replication times differ significantly, most of the major events occur in the same sequence in both cycles. The exceptions

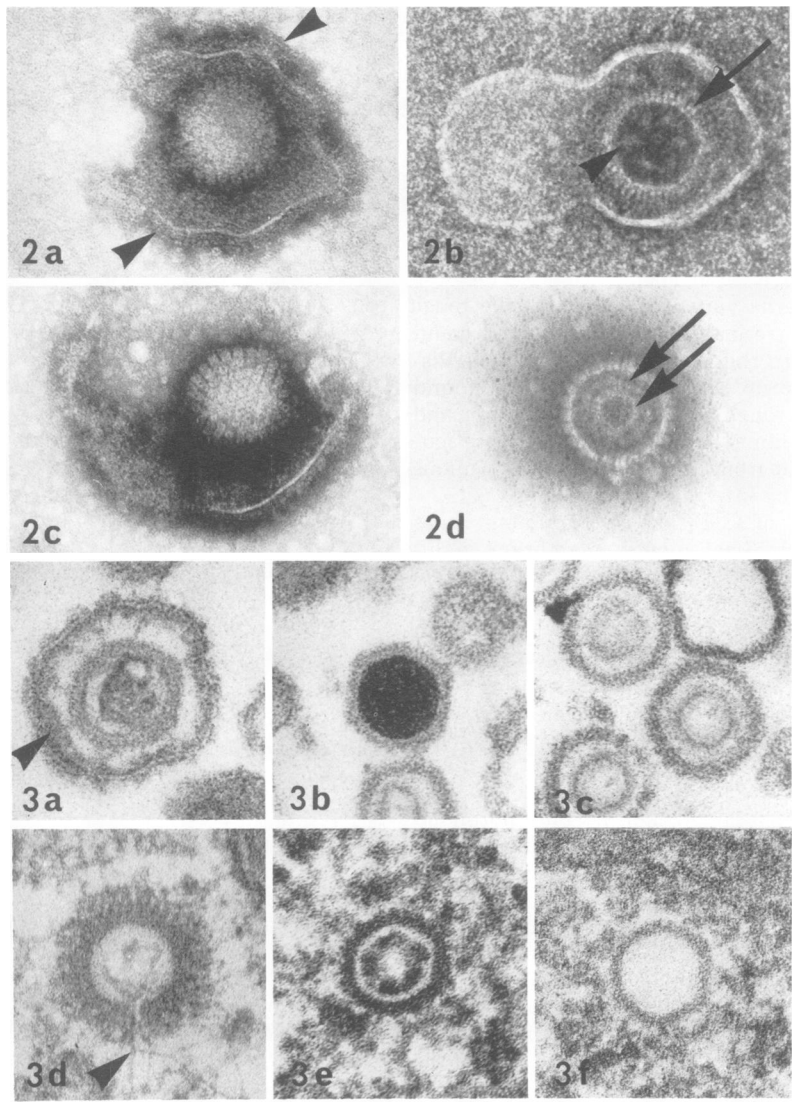


FIG. 2. *Negatively stained HSV and CMV particles. (a) Short 12- to 15-nm projections (arrowheads) sometimes seen on the outer surface of enveloped particles. HSV. (b) Enveloped CMV with individual capsomeres visible at the periphery of the capsid (arrow). The core appears as an aggregate of globular subunits (arrowhead), six or more peripheral and one central. (c) HSV particle showing the hollow polygonal shape of the capsomeres seen end-on. (d) An HSV capsid containing a central core made up of two continuous concentric structures (arrows). (A magnification of ×127,000 was used for a–d.)*

FIG. 3. *HSV and CMV particles in thin section. (a) Enveloped HSV. Note the dense material marginated against the inner surface of the envelope (arrowhead), the capsid, and an apparently ruptured core of intermediate electron density. (b) An unsual HSV capsid completely filled by the electron dense material thought to represent viral nucleoprotein. (c) HSV capsids containing "empty" cores. Note that as in Fig. 2d, the wall of the core seems composed of two layers, the inner here appearing slightly more electron opaque. (d) An unenveloped, cytoplasmic CMV capsid, with fibrillar coat. Traces of a core remain in the interior, along with an unknown material forming an unusual tail-like structure (arrowhead). (e) A CMV capsid within the nucleus. Note the absence of any coat and the beaded appearance of the core. (f) A cytoplasmic HSV capsid lacking a core entirely and which, like all HSV capsids, nuclear or cytoplasmic, is not coated. (A magnification of approximately ×127,000 was used for a–f.)*

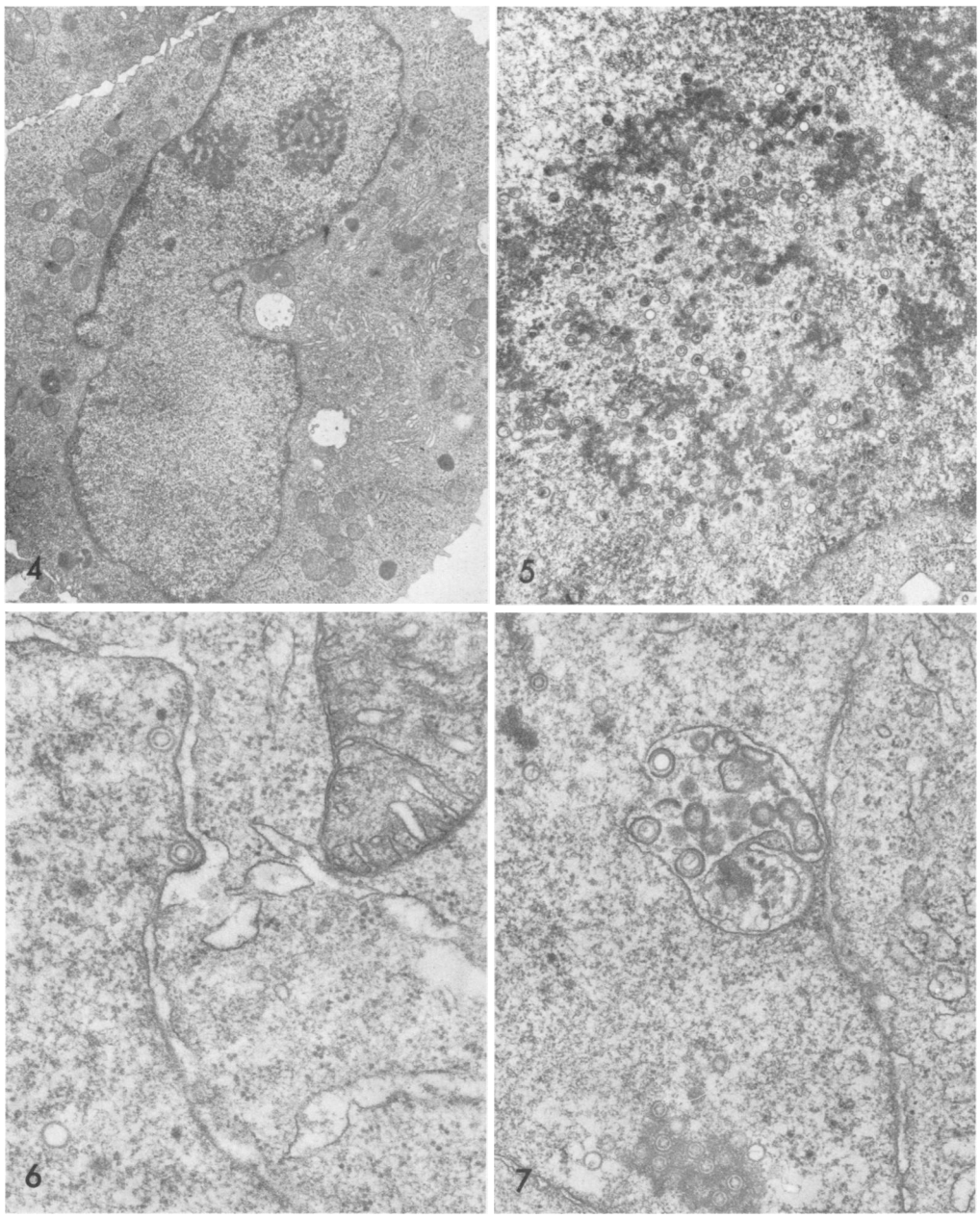


FIG. 4. *A rounded cell 12 h after CMV infection. To the right of the reniform nucleus is a region of the cytoplasm where the accumulation and proliferation of Golgi elements have begun. Magnification ×6,700.*

FIG. 5. *A typical nuclear inclusion 3.5 days after CMV infection. It is composed of irregular anastomosing strands of electron dense material and is in close proximity to most of the developing capsids. Magnification ×13,000.*

FIG. 6. *A single CMV capsid acquiring an envelope by budding through the inner nuclear membrane into the perinuclear cisterna. Three and one-half days postinfection. Magnification ×37,000.*

FIG. 7. *CMV capsid envelopment by budding into a membrane-bounded sac which is presumably continuous with the perinuclear cisterna. Four days postinfection. Magnification ×25,500.*

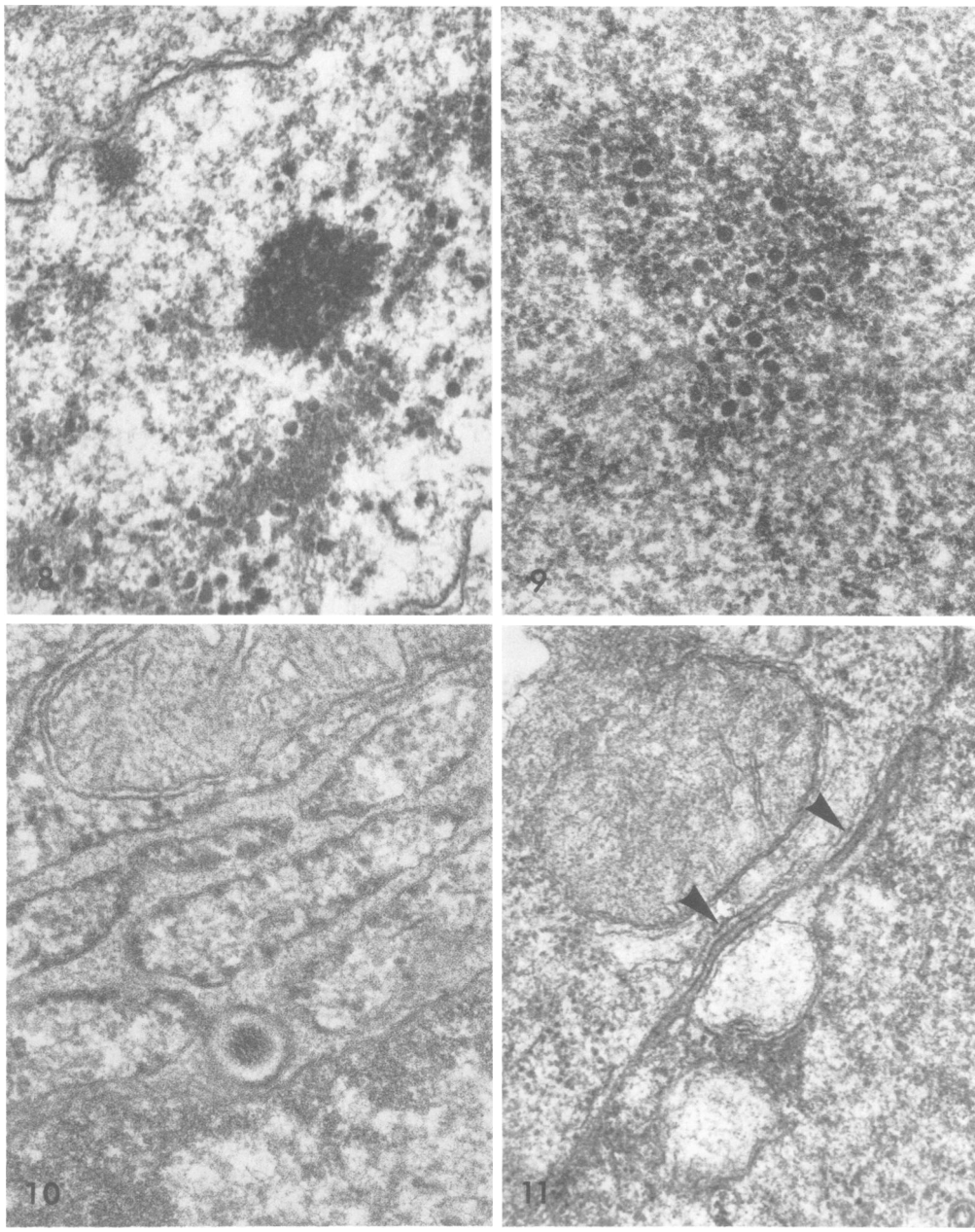


FIG. 8. *A large accumulation of "perichromatin-like" granules in an HSV-infected nucleus. The granules occur in association with heterochromatin and occasionally near characteristic granular aggregates. 2 h postinfection. Magnification ×36,000.*

FIG. 9. *"Perichromatin-like" granules in a nucleus 2 days after infection with CMV. Note the clear region or "halo" separating them from the surrounding heterochromatin. Magnification ×38,000.*

FIG. 10. *A tangential section of an enveloped particle in the perinuclear cisterna near the point of continuity with the rough endoplasmic reticulum. HSV, 8 h postinfection. Magnification ×75,000.*

FIG. 11. *At several points (arrowheads) short segments of reduplicated membranes separate the nucleus (lower right) from the cytoplasm of a cell 4 days after infection with CMV. Magnification ×48,000.*

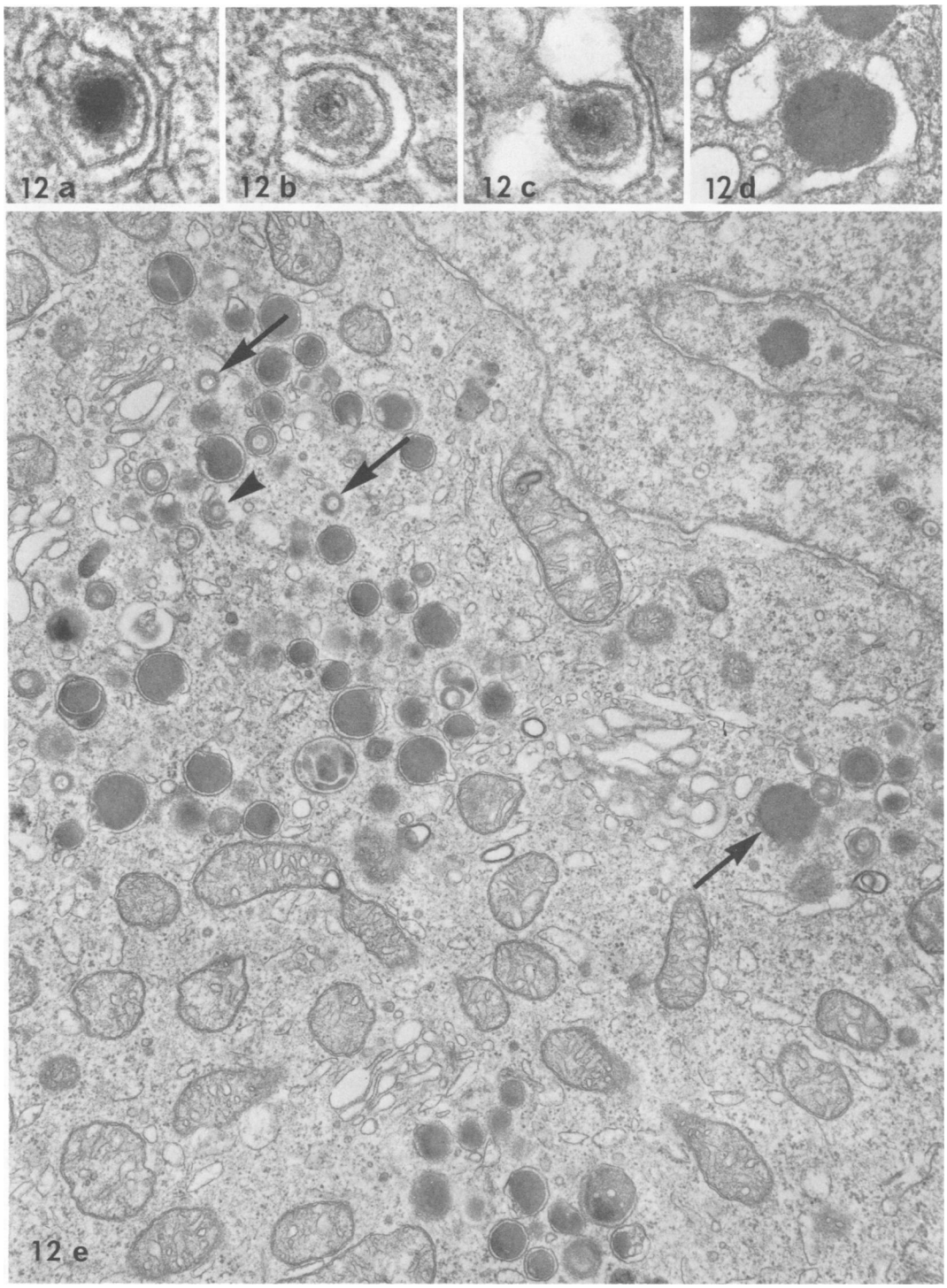


FIG. 12. *Cytoplasmic envelopment in CMV infection. (a–c) Naked CMV capsids budding through one side of a cytoplasmic vacuole. Note that in budding particles the thickness of the capsid coat is significantly reduced. Magnification approximately ×100,000. (d) A typical dense body undergoing cytoplasmic envelopment in a manner similar to viral capsids. Six days postinfection. Magnification ×32,000. (e) An area of cytoplasm rich in Golgi elements and containing several unenveloped, coated capsids (arrows, upper left), one of which (arrowhead) is budding into a small cytoplasmic vacuole. Many dense bodies, both enveloped and unenveloped (arrow, lower right) are also present. Four and one-half days postinfection. Magnification ×24,000.*

TABLE 1. *Comparison of timing of major events in HSV and CMV cycles.*

Event	Onset	
	CMV (days)	HSV (h)
Cell fusion and rounding	0.5	6
Golgi alteration	1	8
Viral eclipse	2	2
Appearance of increased numbers of perichromatin-like granules	2	3
Condensation of the chromatin	Not observed	3
Assembly of the first capsids in the nucleus	3	4
Appearance of the first capsids with dense cores	3.5	5
Envelopment at the nuclear membrane	3.5	6
Appearance of naked capsids in the cytoplasm	4	6
Appearance of dense cytoplasmic aggregates	4	Not observed
First released particles	4	8
Nuclear membrane reduplication	4	8
Envelopment at cytoplasmic membranes	4	8
Cell lysis	7–8	24–48

are those reflecting the reorganization of cellular architecture. These, including cell fusion, cell rounding, and the proliferation and consolidation of the Golgi elements, occur within hours after infection by either virus, and therefore relatively earlier in the longer cycle of CMV.

In addition to these temporal variations there were qualitative differences which may be summarized as follows:

(i) The naked cytoplasmic capsids of HSV, unlike those of CMV, were never coated.

(ii) The cytoplasmic dense bodies (Fig. 12e) characteristic of CMV infection were not found in HSV-infected cells.

(iii) The skein-like nuclear inclusions of CMV-infected cells were not observed after infection with HSV. Nuclear inclusions were not common until late in the replication cycle, when two characteristic types appeared (Fig. 15, 16).

(iv) By 3 h postinfection with HSV there was a marked condensation and margination of the chromatin, which persisted throughout replication (Fig. 17, 18). Such condensation was entirely absent with CMV.

(iv) By 3 h postinfection with HSV there was a marked condensation and margination of the chromatin, which persisted through replication (Fig. 17, 18). Such condensation was entirely absent with CMV.

(v) Extensive membrane changes followed infection by this strain of HSV. These included the following: (i) cell fusion with formation of large polykaryocytes often containing 12 or more nuclei; (ii) reduplication of the nuclear membrane, with two or more extra membrane pairs over much of the periphery of the highly lobulated nucleus; and (iii) proliferation and extensive morphological alteration of the membranous elements in the Golgi region. With respect to this last point, by 8 h postinfection the typical vesicles and saccules of the Golgi had been almost entirely replaced by two new elements, namely, concentric cisternae bounded by smooth membranes (Fig. 18) and large smooth-surfaced vacuoles arranged in characteristic clusters (Fig. 17, 18). Cytoplasmic envelopment of naked HSV capsids often occurred at these large vacuoles.

DISCUSSION

Since both viral cycles described above were carried out under standardized conditions, the observed differences between the two presumably reflect differences in the genetic makeup of the viruses rather than variation in the experimental system.

To the extent that other strains of HSV or CMV are related to those used here, they should show similar distinctive patterns of replication when compared in the same cell system. Indeed, earlier investigations with other strains have shown similar patterns with regard to chromatin margination and condensation by HSV (15); lack of condensation with CMV (5, 10, 13, 18); extensive membrane changes with HSV, i.e., either polykaryocytosis or extensive cell rounding (12, 16); and only minimal effects with CMV (13, 18). Nor are the patterns of replication we observed limited to laboratory strains. Thus, extensive polykaryocytosis has been observed not only with adapted strains of HSV, but also with recently isolated virus (1, 7, 22), and even in biopsy material (2, 20, 21). A similar concordance between many of the major effects of an adapted strain and a fresh isolate was reported recently for CMV (11).

With regard to viral morphology, we observed many of the same features reported by others for HSV (15, 31) and CMV (13, 26). The double-walled structure of the HSV core resembles that described for the Lucké virus (27), whereas the beaded or globular appearance of the CMV core has been illustrated by a number of published micrographs (5, 10, 11, 13). We did not find evidence with either HSV or CMV for a toroidal structure of the core, such as reported recently for HSV (9). This was true even in late stages of

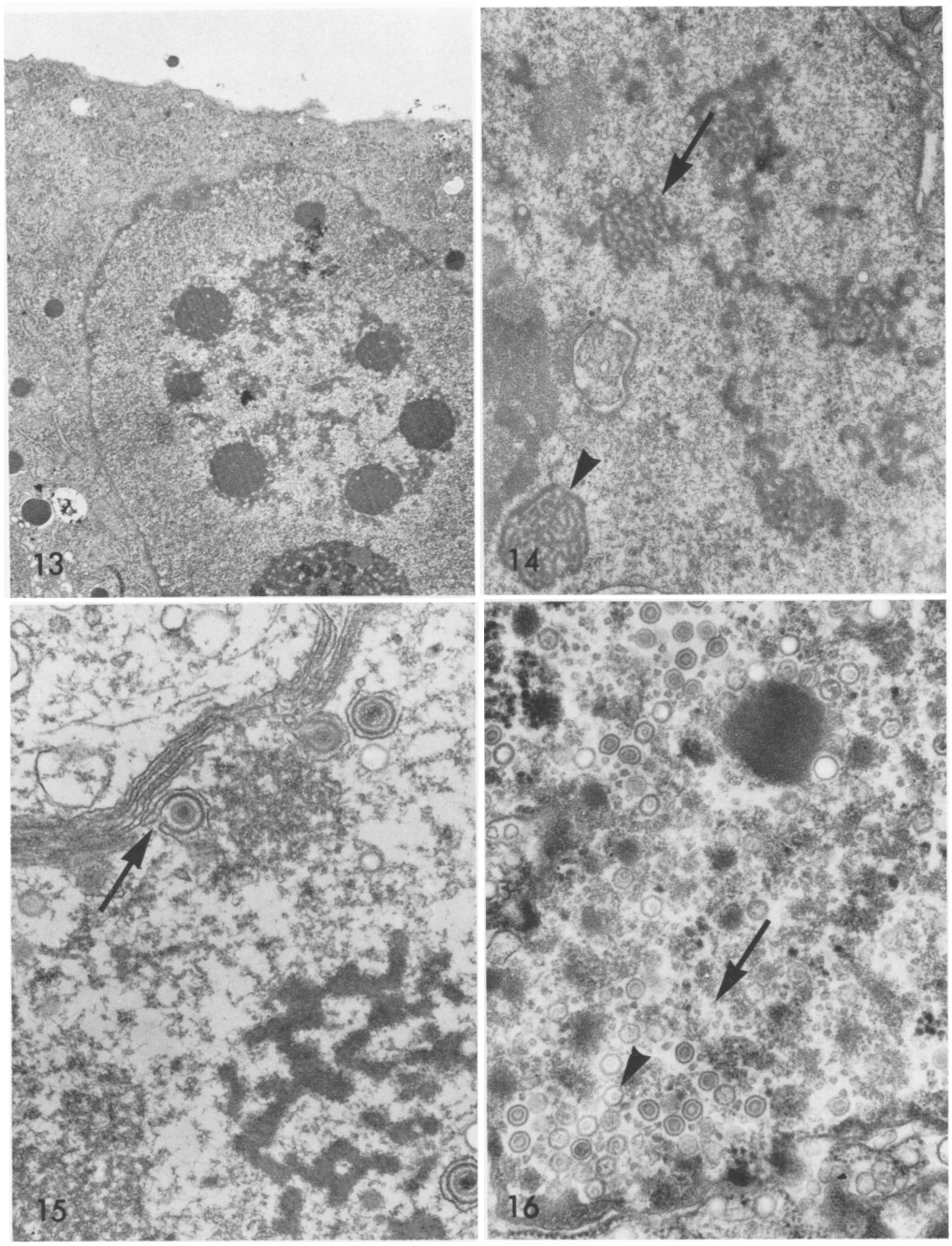
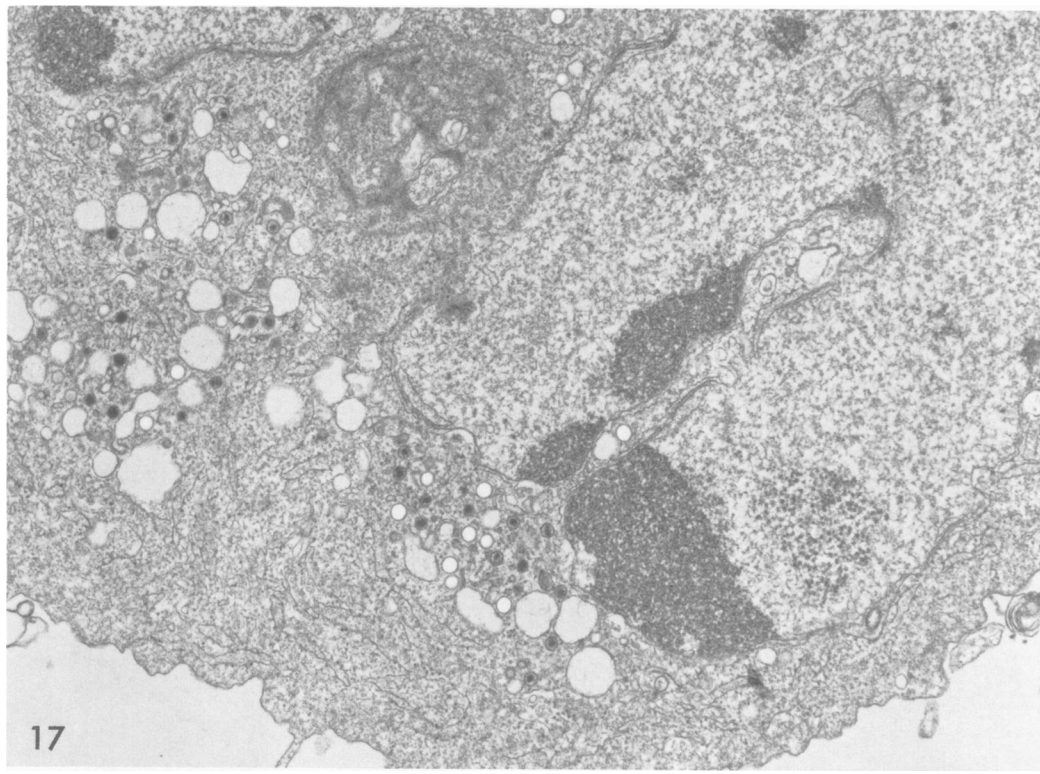


FIG. 13. *A late CMV inclusion composed of multiple round masses, associated at their periphery with viral capsids and with the remnants of the earlier skein-like inclusions, and occupying an area of reduced electron opacity in the center of the nucleus. A rim of nucleoplasm separates the inclusion from the nuclear membrane and from the segregated nucleolus at the lower right. Six days postinfection. Magnification ×6,300.*

FIG. 14. *Several inclusions resembling granules or short fibrils (arrow) and one more compact fibrillar form (arrowhead) are closely associated with the remnants of the skein-like CMV nuclear inclusion. Six days postinfection. Magnification ×18,000.*

FIG. 15. *A late HSV inclusion is present in the lower right. Note also the extensive reduplication of the nuclear membrane as well as the viruses acquiring an envelope at their inner face (arrow). Twelve hours postinfection. Magnification ×34,000.*

FIG. 16. *A round inclusion body (upper right) common in late HSV infection. These were scattered singly in the nucleoplasm rather than in groups, and showed no trace of a fibrillar substructure. Note also the numerous small, ring-like structures free in the nucleoplasm (arrow). Although smaller than normal viral cores, several appear to be surrounded by typical capsids (arrowhead), suggesting they may be the result of aberrant core assembly in late infection. Twenty-four hours postinfection. Magnification ×36,000.*



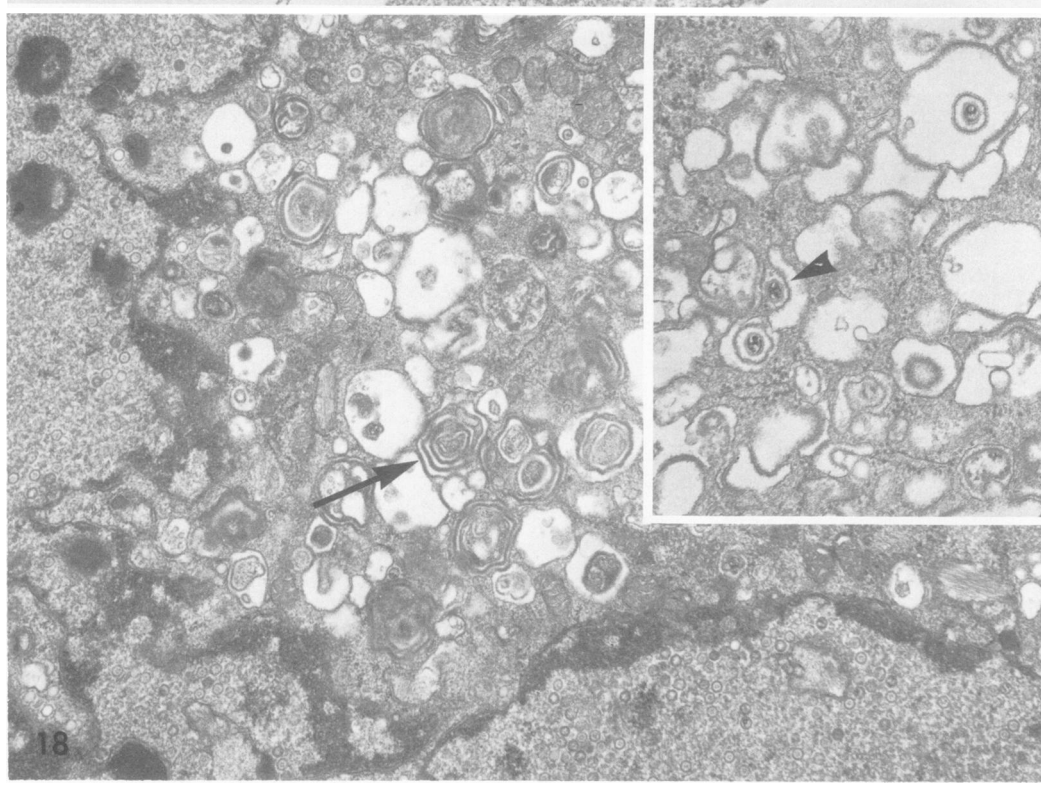


FIG. 17. *An HSV-infected cell showing long stretches of nuclear membrane reduplication as well as condensation and margination of the chromatin into several large masses. Within the cytoplasm the typical Golgi elements have been replaced by large, apparently empty vacuoles; and numerous unenveloped capsids, all lacking coats, are present. Ten h postinfection. Magnification ×75,500.*

FIG. 18. *An HSV-infected cell in which the typical Golgi membranes have been replaced by concentric arrays of membrane-bounded cisternae (arrow), and large vacuoles. Often naked cytoplasmic capsids became enveloped by budding into the latter (inset). Ten h post infection. Magnification ×14,000; inset, ×21,000.*

infection when bizarre core forms often appear (15).

In a system identical to our own, i.e., CMV strain AD169 growing in WI-38 cells, other investigators were not able to document capsid envelopment at the nuclear membrane (11). However, we regularly observed the full process of CMV envelopment at the nucleus, as have other workers (13).

Despite the observation of envelopment at the nuclear membrane, other capsids of both HSV and CMV are consistently found unenveloped in the cytoplasm. Unenveloped cytoplasmic CMV capsids were coated, whereas those of HSV were not. These results differ from those in an early report (5) which described CMV capsids in monkey kidney cells as uncoated. We found coating to be a strictly cytoplasmic phenomenon, in agreement with the observation of Stackpole (27) for Lucké virus.

Our studies revealed only one method of nonlytic viral release, namely, fusion between a vacuole carrying an enveloped virus and the plasmalemma, a process which has been aptly described as reverse phagocytosis (14). Other investigators (19) have described a tubular system continuous at one end with the perinuclear cisterna, and with the extracellular space at the other, which might serve as a direct avenue of viral release in HSV-infected HEp-2 cells. Despite numerous attempts to observe such tubules by through sectioning of in situ embedded monolayers in a variety of selected orientations, we saw no evidence for them in either HSV- or CMV-infected cells.

We did observe the formation of cytoplasmic dense bodies in CMV-infected cells in a manner reported by others (10, 11, 13, 18). However, on the basis of cytochemical data reported elsewhere (manuscript in preparation), we feel that the common assumption that these represent lysosomes is probably erroneous.

With regard to the timing of the two cycles, our results agree well with those of previous authors for HSV (15, 24) and with some of the more limited data available for CMV (13, 18). However, we were not able to identify any limiting steps in CMV reproduction which might explain the much longer cycle time. As we have shown (manuscript in preparation), the limitation does not appear to involve viral adsorption or penetration, since capsids of HSV and CMV entering the cell from the inoculum both reach the periphery of the nucleus in approximately the same time.

Finally, it should be pointed out that although we have defined several criteria for discriminating between HSV and CMV infections in this system, we do not as yet understand the biochemical basis for any of these distinctions. Especially intriguing is the failure of CMV to cause condensation of host cell chromatin, since this may reflect an effect on host cell macromolecular synthesis which is different from that of most herpesviruses (12, 17). High-titer CMV inocula, which can now be obtained in a variety of ways (4, 10, 25) should make this and other similar questions accessible to investigation by biochemical methods.

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