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

III. Cytochemical Localization of Lysosomal Enzymes in Infected Cells

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Cytochemical localization of the lysosomal enzymes acid phosphatase and arylsulfatase in cells infected by herpes simplex virus (HSV) or human cytomegalovirus (CMV) showed the following interactions between viruses and host cell lysosomes: (i) many enveloped progeny viruses were located within cytoplasmic vacuoles containing lysosomal enzyme activity; (ii) naked cytoplasmic capsids appeared to acquire an envelope by budding directly into lysosomes; and (iii) many of the cytoplasmic dense bodies that are characteristic of CMV-infected cells and are thought to represent noninfectious aggregates of CMV structural proteins (I. Sarov and I. Abady, Virology **66**:464-473, 1975) also acquired a limiting membrane by budding into lysosomes. Autophagy of other cytoplasmic elements was not observed, suggesting that there is some specificity involved in the association of viral particles and CMV dense bodies with lysosomes. Despite the presence of potentially destructive hydrolases, there was little evidence of significant morphological damage to intralysosomal viruses, and high titers of infectious particles were released into the medium. It would therefore appear that significant levels of HSV and CMV infectivity normally persist even though many progeny particles are directly exposed to lysosomal enzymes.

Degradation by lysosomal enzymes has been proposed as a mechanism for the inactivation of cell-associated herpesviruses (2, 4). Consistent with this hypothesis are the observations that during normal replication varicella-zoster virus (VZV), a highly cell-associated herpesvirus, appears to undergo morphological degradation within cytoplasmic vacuoles (2) and that acid phosphatase (AP) activity can be cytochemically localized within such VZV-containing vacuoles (4). In contrast, with other members of the herpes group, such as herpes simplex virus (HSV) or laboratory-adapted strains of human cytomegalovirus (CMV), high titers of infectious cell-free virus are released into the medium, and viral particles generally appear morphologically intact within cytoplasmic vacuoles (8, 10, 12, 19). Yet, at least in the case of HSV, it has been shown that the virions in vitro are in fact susceptible to inactivation by purified AP and that inhibitors of AP activity will prolong the survival of the virus in culture fluids (1). The present cytochemical study was therefore undertaken to determine whether HSV or CMV particles are normally exposed to lysosomal hydrolases during the course of their maturation.

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MATERIALS AND METHODS

Cells. WI-38 cells (American Type Culture Collection), human diploid fibroblasts, were propagated in minimum essential medium containing 10% fetal calf serum. Infected cells were maintained on medium containing 2% fetal calf serum.

Virus. Cell-free stocks of HSV, strain HF, and CMV, strain AD-169 (American Type Culture Collection), were prepared by rapidly freezing and thawing infected cells and were titrated by plaque assay (22, 23). For enzyme localization studies, subconfluent monolayers in 35-mm petri dishes were infected as reported previously (19) at a multiplicity of 5 PFU/cell. At various times postinfection (p.i.), portions of culture medium were titrated to demonstrate productive, one-step infection.

Enzyme localization. In separate experiments, AP or arylsulfatase (AS) activity was localized in HSV- or CMV-infected cells. AP was localized by a modification of the method of Gomori (5), using in situ fixation for 15 min with 1.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) and overnight rinsing in buffer containing 5% sucrose. Cytochemical incubation was carried out for 40 min in a 37°C water bath, using a filtered reaction mixture containing 5 mg of CMP

(Sigma Chemical Co.) as substrate (13), 2.4 ml of deionized water, 2.0 ml of 0.1 M acetate buffer (pH 5.0), and 0.6 ml of 1% aqueous lead nitrate. After incubation, the cultures were rinsed in deionized water and then in 5% aqueous ammonium sulfide, making the reaction product visible by light as well as electron microscopy. The samples were then fixed for 40 min with 1% OsO_4, dehydrated through alcohol, embedded in a thin layer of Epon, and polymerized at 60°C. Propylene oxide was avoided due to its solubilizing effect on plastic petri dishes. Selected areas were cut from the polymerized Epon disks with a jewelers' saw, mounted on Epon blocks with epoxy glue, sectioned, stained with uranyl acetate and lead citrate, and carbon coated. Sections were examined in a Philips 200 or a Siemens Ia microscope.

AS activity was demonstrated by a modification of the technique of Hopsu-Havu et al. (7) and Hoffstein et al. (6), using in situ fixation as above and a reaction mixture containing 120 mg of *p*-nitrocatechol sulfate (Sigma Chemical Co.) as substrate, 3 ml of deionized water, 9 ml of 0.1 M acetate buffer (pH 5.5), 3 ml of either 5% barium chloride or 8% aqueous lead nitrate, and 1.7 ml of dimethyl sulfoxide. The mixture was filtered and adjusted to pH 5.0 to 5.5 with 0.2 M acetic acid. Incubation was for 90 min in a 37°C water bath, with further processing as for AP.

Controls included infected and uninfected cultures incubated without substrate and uninfected cultures incubated with substrate. For HSV, samples were taken at hourly intervals from 0 to 6 h p.i. and also at 8, 10, 12, and 24 h. For CMV, a more slowly replicating virus, samples were taken at 12-h intervals from 0 to 7.5 days p.i.

RESULTS

Controls. Uninfected cells incubated for AP showed reaction product in the cisternae of the Golgi complex, within some profiles of rough endoplasmic reticulum, and in lysosomes of various morphological types. In tangential section (Fig. 1), many of the AP-positive cisternae appeared fenestrated in much the same way as described by Novikoff et al. for the GERL complex (Golgi-endoplasmic reticulum-lysosome complex) in rat spinal ganglia (14). The remaining cytoplasmic organelles, as well as the nucleus and the perinuclear cisterna, were always free of reaction product. AS reaction product was largely limited to the lysosomes and was only rarely found in the endoplasmic reticulum or the Golgi. As expected, infected and uninfected cultures incubated without substrate were always negative for reaction product.

In infected cultures, portions of medium sampled at various times p.i. and titrated by plaque assay showed that the conditions of infection

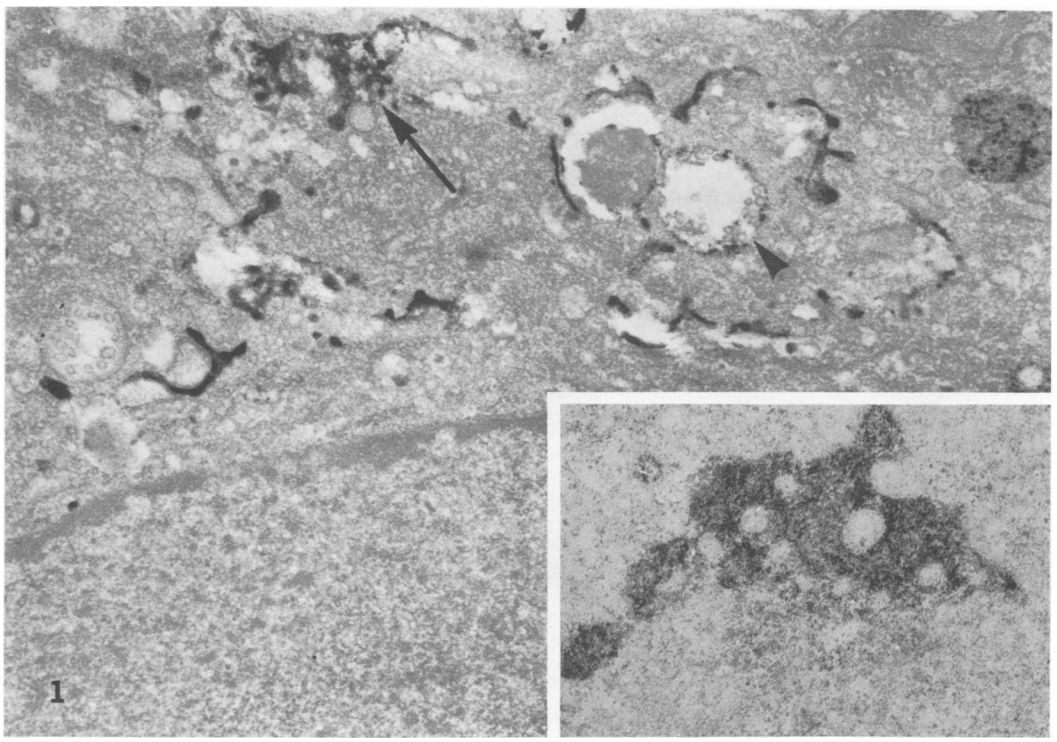

FIG. 1. *Uninfected WI-38 cell, showing AP reaction product in a variety of cytoplasmic vacuolar elements including multivesicular bodies (arrowhead) and membranous cisternae of the GERL complex. Where sectioned tangentially, the latter show characteristic fenestrations (arrow and inset).* ×16,700; *inset,* ×70,500.

had led to one-step growth cycles for both HSV and CMV (Fig. 2). Electron microscopy revealed that an average of 92 to 94% of the cells had been initially infected and that the infection proceeded with little evidence of asynchrony.

HSV-infected cells. HSV capsids began to assemble in the nuclei of infected cells by 4 h p.i., and by 5 h capsids were observed budding into the perinuclear cisternae. Neither AP nor AS reaction product was ever associated with intranuclear capsids or with the enveloped intracisternal particles.

Intralysosomal viral particles were observed throughout the HSV cycle. In samples fixed 1 to 4 h p.i., rare enveloped viruses and naked capsids were located within AP- or AS-positive cytoplasmic vacuoles. These intralysosomal particles presumably represent part of the inoculum. Beginning at 6 h p.i., we observed the onset of the logarithmic phase of infectious virus replication as determined by plaque assay (Fig. 2) and, correlated with this, a significant increase in the number of intralysosomal viral particles (Fig. 3 and 4). Many of these presumably represent progeny virus. The great majority of these intra-

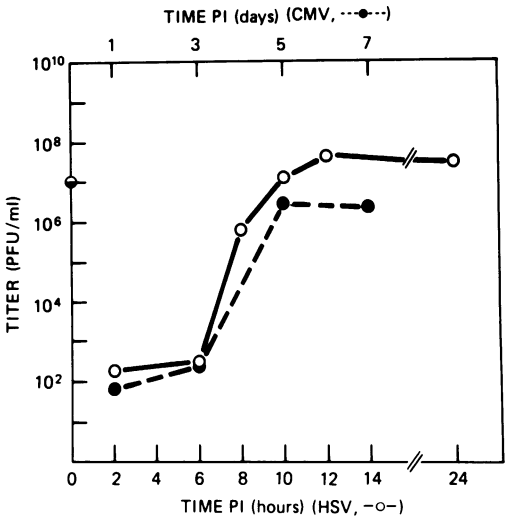

FIG. 2. *Growth curves for HSV (○) and CMV (●) as determined by plaque assay of culture fluids sampled at the designated times. The point at zero time represents the titer of the inocula in PFU per milliliter. Each petri dish received 0.1 ml of the appropriate inoculum.*

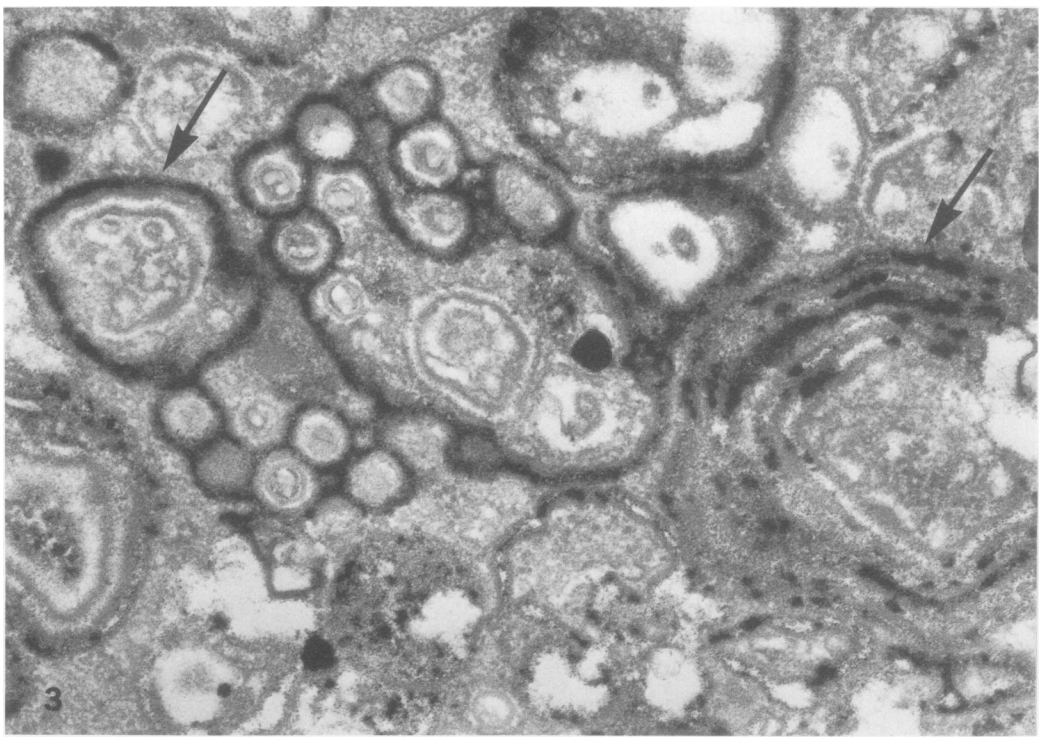

FIG. 3. *Enveloped HSV particles directly exposed to AP activity. Reaction product surrounds each particle but has not penetrated the viral envelope. Also present are concentric arrays of membrane-bound channels, many of them AP positive (arrows), which appeared to be derived from the Golgi apparatus. These and other modifications of the Golgi elements were common in HSV- but not in CMV-infected cells. 12 h p.i. ×46,800.*

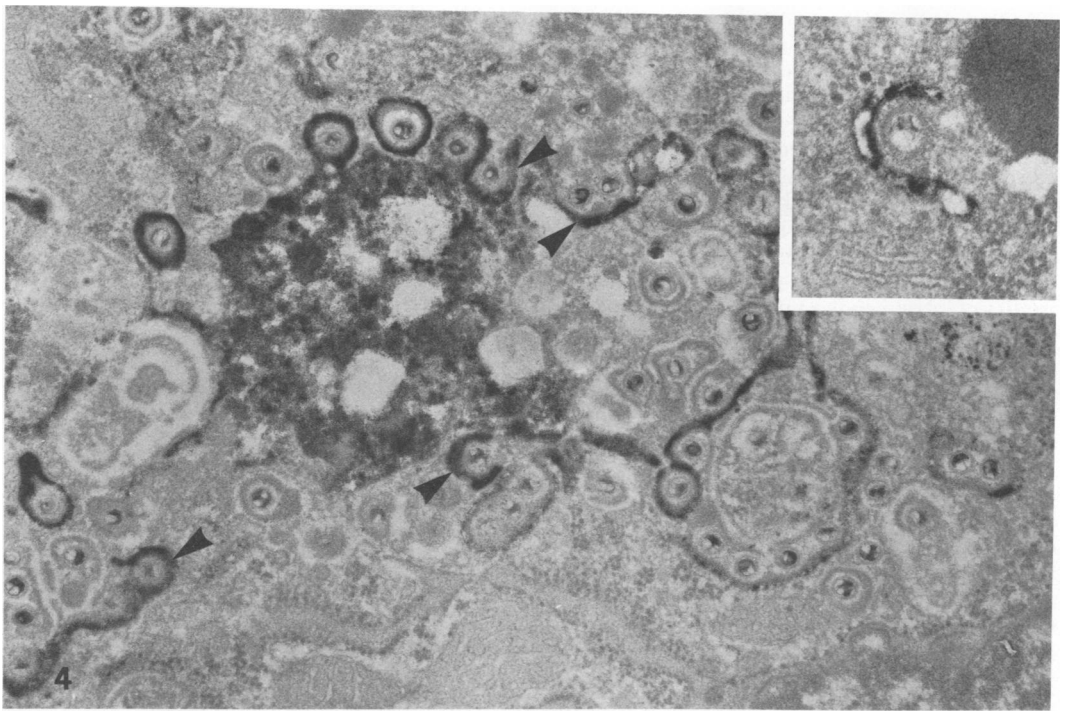

FIG. 4. *Numerous naked HSV capsids (12 h p.i.) acquiring an envelope in the cytoplasm by budding into vacuoles and membrane-bounded cisternae containing AP enzyme activity (arrowheads). (Inset) Higher magnification showing the same envelopment process in a CMV-infected cell. The capsids contain variable amounts of viral DNA and associated proteins. 5.5 days p.i. ×28,500; inset, ×55,200.*

lysosomal particles were enveloped virions. At 12 h p.i., 50% or more of the enveloped particles in AP-reactive cells were located within lysosomes. With AS the percentage was somewhat lower. Since even brief exposure to glutaraldehyde can decrease enzyme activity, it is likely that we are underestimating the true extent of viral exposure to lysosomal enzymes.

Despite the direct exposure of intralysosomal viral particles to AP or AS activity, viral morphology appeared to be relatively intact (Fig. 3 and 4). For example, reaction product was rarely observed between the envelope and the capsid, suggesting that major discontinuities in the envelope were uncommon. Likewise, naked intralysosomal capsids suggestive of envelope disruption were rarely encountered. There was no evidence at all of the widespread degradation seen in VZV infections (2, 4).

Beginning at 4 to 6 h p.i., we observed the appearance of unenveloped or naked capsids in the cytoplasm. These may represent particles released from the nucleus through localized discontinuities in the nuclear membranes of infected cells. AP or AS reaction product was never associated with naked capsids free in the hyaloplasm. However, naked capsids frequently appeared to acquire a membrane by budding into crescent-shaped cytoplasmic vacuoles, and many of these vacuoles were positive for AP or AS activity (Fig. 4). In any given sample a progression of images could be observed, indicative of successive stages in the budding process. The budding phenomenon continued through the latest stages of infection. It would therefore appear that the process of cytoplasmic envelopment can sometimes involve the budding of naked capsids into lysosomes, such that the resulting enveloped particle is directly exposed to lysosomal hydrolases.

CMV-infected cells. In cells infected with this more slowly replicating herpesvirus, enveloped and unenveloped particles from the inoculum had disappeared by 1.5 days p.i., whereas progeny particles did not begin to assemble in the nucleus until 3 days p.i. There was, therefore, a clear period of morphological viral eclipse lasting 24 h or more, between the disappearance of particles derived from the inoculum and the appearance of progeny particles in the nucleus.

At 3 days p.i. and later, the relationship between the progeny CMV particles and lysosomal

enzyme activity was essentially as described for HSV infection. Intranuclear CMV capsids and enveloped particles in the perinuclear cisternae were never associated with AP or AS activity, but in the cytoplasm naked capsids appeared to bud into lysosomes during the process of cytoplasmic envelopment (Fig. 4, inset). Again, despite direct contact with lysosomal enzymes, intralysosomal particles rarely showed disruption of the viral envelope or other evidence of viral breakdown. This agrees with our earlier studies of intravacuolar virus by routine electron microscopy (19).

As previously reported (19), homogeneous, electron-opaque dense bodies developed in the cytoplasm of CMV- but not HSV-infected cells (Fig. 5 and 6). When these dense bodies first appeared in the cytoplasm between 3 and 3.5 days p.i., they had no limiting membrane. However, like naked cytoplasmic viral capsids, they could acquire an envelope by budding into crescent-shaped vacuoles, many of which were AP or AS positive (Fig. 6). Within these lysosomal vacuoles, reaction product was commonly localized in the vacuolar space between the lysosomal membrane and the limiting membrane of the dense body, but was rarely observed within the enveloped dense body itself (Fig. 5 and 6). This suggests that, like the viral envelope, the membrane surrounding a dense body often remains largely intact within lysosomes. No alternative method of dense body envelopment other than budding was observed. Large dense bodies (greater than 500 nm in diameter), instead of budding, were often closely surrounded by several small vacuoles. Many of these small satellite vacuoles were AP or AS positive (Fig. 5).

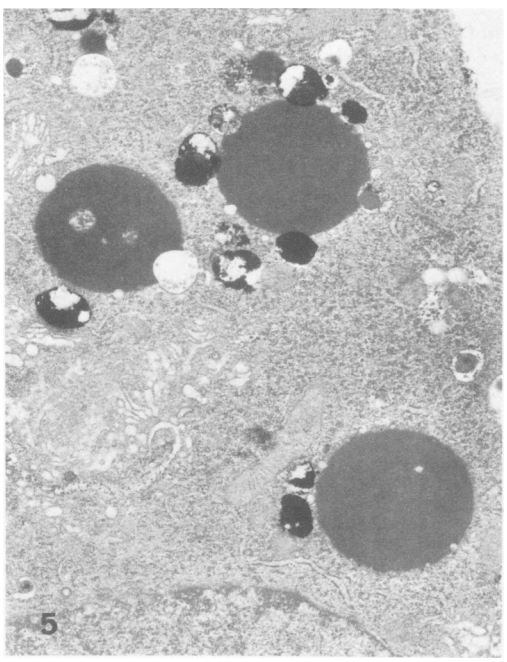

FIG. 5. *Several large, unenveloped dense bodies in a CMV-infected cell are surrounded by a variety of small satellite vacuoles, many of which are positive for AP activity. The dense bodies themselves contain no reaction product. 5 days p.i. ×7,400.*

Both CMV and HSV were released from AP- or AS-positive vacuoles at the cell surface, apparently via a process of exocytosis, implying a continuous release of enzyme into the medium during their infectious cycles.

DISCUSSION

We have demonstrated that many intravacuolar HSV and CMV particles, like those in VZV-infected cells (4), are actually located within lysosomes and are therefore directly exposed to lysosomal hydrolases. Furthermore, naked cytoplasmic HSV and CMV capsids appear to acquire an envelope by budding directly into lysosomes. That this is a true budding process and not simply a chance juxtaposition of viral capsids and crescent-shaped lysosomes is inferred from the fact that in a given sample we could observe what appeared to be successive stages in budding. In fact, we as well as other investigators (8, 10, 12, 19) have previously published micrographs illustrating the presumed stages of HSV and CMV budding in cells studied by conventional electron microscopy. Furthermore, the images of viral budding are strikingly similar to those seen with unenveloped dense bodies, where a true budding process appears to be the only means of envelopment. It would appear, therefore, that the images we observe do represent cytoplasmic envelopment of naked viral capsids via budding and, furthermore, that the vacuoles involved in the envelopment of some capsids are lysosomal in nature.

It appears likely that the majority of the intralysosomal particles seen after 6 h with HSV or 3 days with CMV represent progeny virus and not residual particles from the inoculum. This is based on (i) the large numbers of intralysosomal particles often observed per cell section (Fig. 3 and 4), despite the relatively low input multiplicities used; (ii) the appearance of large numbers of intralysosomal particles at approximately the same time that progeny virions are first detected by plaque assay; (iii) the rarity of naked capsids within lysosomes, despite the fact (18) that naked capsids are common in inocula prepared by freezing and thawing infected cells; and (iv) the clear 24-h period of morphological

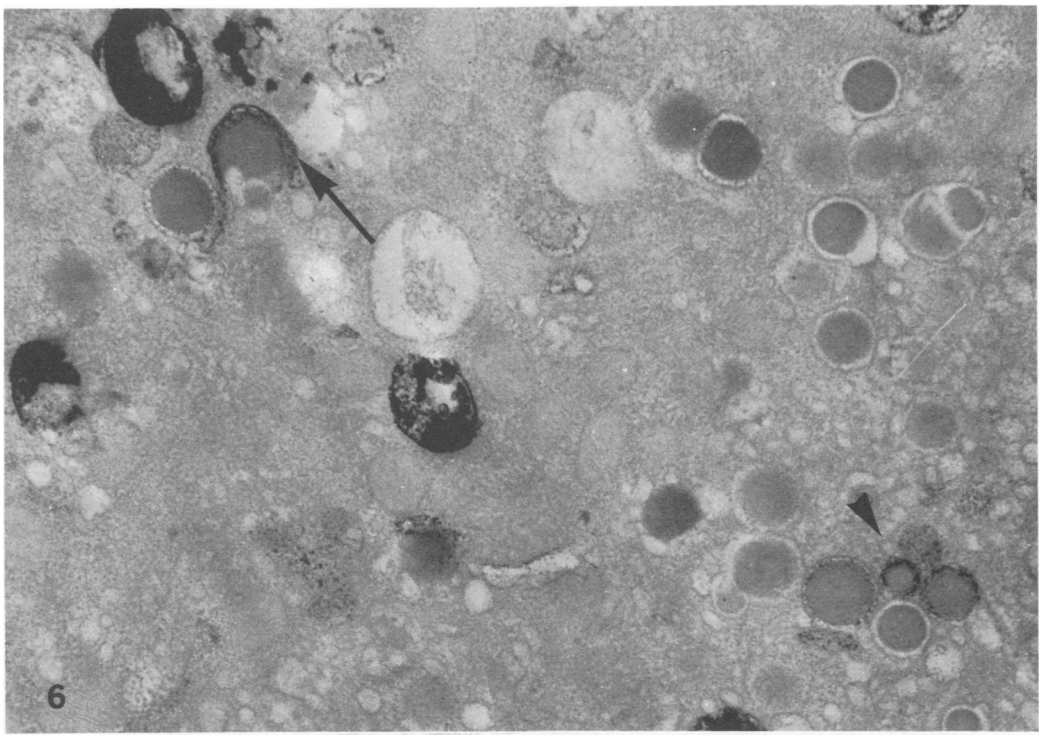

FIG. 6. *Several enveloped CMV dense bodies are located within AP-positive vacuoles (arrowhead), while one unenveloped dense body (arrow) buds into a crescent-shaped vacuole containing AP reaction product. Compare with Fig. 4 (inset), showing the cytoplasmic envelopment of a naked CMV capsid. 4.5 days p.i. ×20,000.*

eclipse observed between the disappearance of the viral inoculum and the appearance of progeny virus in CMV-infected cells. Similarly, the synchrony of the infection, as shown in Fig. 2 and as verified by our electron microscope observations, makes it unlikely that we are observing the early stages of viral penetration into previously uninfected cells. Indeed, the majority of cells containing intralysosomal virus also showed evidence of advanced viral infection as judged by the presence of capsid assembly in the nucleus.

In VZV-infected cell cultures, intralysosomal particles show evidence of morphological degradation (2, 4), and virtually all released particles are noninfectious (2, 20, 21); yet our observations show that intralysosomal HSV and CMV appear largely intact and that high titers of infectivity can be recovered from the medium. Furthermore, working with conventional preparations for electron microscopy, where the preservation of ultrastructural detail is superior to that found in samples incubated for electron microscope cytochemistry, numerous investigators including ourselves (3, 8–12, 15, 16, 19) have previously reported morphologically intact intravacuolar HSV and CMV and have correlated this appearance with high yields of infectious virus.

If HSV and CMV progeny particles are frequently exposed to lysosomal enzymes, and if the virions are susceptible to lysosomal inactivation as suggested by the available data on HSV (1), how then can the characteristically high yields of infectious virus be explained? The following hypotheses would appear tenable.

(i) The infectious virions may represent a small population that egresses from infected cells in nonlysosomal vacuoles. In most cells we observed virus-containing vacuoles that were negative for acid hydrolase activity, but it is difficult to determine whether these are truly nonlysosomal in nature or whether glutaraldehyde fixation may have inactivated the AP or AS activity they originally contained. Also, this hypothesis would not explain why there is so little morphological evidence of viral degradation within lysosomes.

(ii) HSV and CMV may retain their infectivity despite exposure to lysosomal hydrolases if their transit time across the cytoplasm is rapid, so that the virions are only briefly exposed to lysosomal enzymes and are released from the cell

before inactivation can occur.

(iii) The origin of the viral envelope may influence the susceptibility of the virion to intralysosomal inactivation. Our data suggest that HSV and CMV virions can acquire their envelope not only at the inner nuclear membrane, but also from lysosomal membranes. It is possible that the lysosomal membranes are inherently more resistant to degradation by acid hydrolases and that they, therefore, protect cytoplasmically enveloped particles against intralysosomal inactivation. With our present morphological methods, we cannot distinguish among these possible explanations.

The dense bodies that we observed in CMV-infected cells have been reported previously (3, 8–11, 15, 16, 19), and their envelopment via budding has also been noted (3, 8, 10, 19). They do not appear in HSV-infected cells (19). Whereas earlier investigations suggested that dense bodies might be lysosomes (11, 15, 16), Craighead and co-workers, using electron microscope cytochemistry (3), observed no AP reaction product within them. This result is supported by our present findings with both AP and AS. However, whereas these investigators observed dense bodies budding into nonlysosomal vacuoles only (3), we have repeatedly observed dense bodies budding into AP- and AS-positive vacuoles (Fig. 6). We conclude that dense bodies are not themselves lysosomes, but that they can bud into lysosomes in the same manner as do naked capsids, acquiring an envelope in the process.

It has been reported that dense bodies are composed largely of CMV structural proteins (17) that apparently never assemble to produce typical viral particles. The fact that dense bodies and naked capsids both bud into lysosomes, whereas other cytoplasmic components do not, suggests that budding may be the result of some specific affinity between the lysosomal membrane and one or more of the CMV structural proteins common to capsids and dense bodies. Such an affinity might be the basis for the apparent specificity of the interaction between lysosomes and viral particles in infected cells. Finally, the failure of dense bodies to appear in HSV-infected cells may imply that viral assembly is a more efficient process for HSV than for CMV.

In summary, our data suggest that there is a close and apparently specific interaction between lysosomes and viral particles in HSV- and CMV-infected cells, but that despite direct exposure of many progeny virions to potentially destructive acid hydrolases, there is little morphological evidence of viral degradation within lysosomes, and high titers of infectious virus still persist. The inclusion of progeny viruses within lysosomes may be a general characteristic of the herpesvirus group, since it has also been reported for VZV by Gershon and co-workers (4). If this is so, then what remains to be determined is why herpesviruses such as HSV, CMV, and VZV, although apparently exposed in a qualitatively similar fashion to lysosomal enzymes, show such disparate patterns of infectivity and inactivation.

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