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

II. An Ultrastructural Study of Viral Penetration

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An electron microscope study was carried out on the early minutes of herpes simplex virus (HSV) and cytomegalovirus (CMV) penetration into WI-38 cells. Both HSV and CMV entered cells either by fusion of the viral envelope with a limiting cell membrane, or via phagocytosis. Both fusion and phagocytosis occurred within 3 min after the initiation of penetration. After fusion, the naked capsids of CMV free in the cytoplasm became coated with a fine, fibrillar material. CMV capsids thus coated retained a well-defined and easily identifiable morphology until the eclipse of visible viral particles between 1 and 1.5 days postinfection. In contrast, naked HSV capsids free in the cytoplasm were never coated. Rather, within minutes after penetration, they assumed a rounded, less regular outline, and were no longer detectable by 90 to 120 min postinfection. The free naked capsids of both viruses appeared to migrate across the cytoplasm toward the nucleus and to become located near nuclear pores. Both HSV and CMV capsids reached the nucleus as early as 5 min after the initiation of penetration. No further interaction with the nucleus could be documented. Particles were also consistently identified in the Golgi region. Phagocytosed particles generally remained within phagosomes, where they appeared to be degraded. However, stages were identified in what is believed to be the escape of enveloped viruses from phagosomes into the cytoplasm via fusion of their envelope with the phagosomal membrane.

Several previous reports have dealt with the morphological aspects of herpes simplex virus (HSV) penetration into tissue culture cells (4, 6, 9, 10, 13, 16, 17). They have established that HSV enters cells either via phagocytosis or fusion of its envelope with the plasmalemma. The naked capsids which are free in the cytoplasm, presumably as a result of fusion, migrate rapidly across the cytoplasm toward the nucleus and become disassembled within a relatively short time postinfection. No interaction of recognizable viral components with the nucleus has been observed, but the viral DNA has been found by autoradiography (10) to arrive in the nucleus between 15 and 30 min postinfection. In contrast to the above, relatively little data is available for cytomegalovirus (CMV) (12).

HSV is a representative of the rapidly replicating herpesviruses, whereas CMV requires a much longer time for production of infectious progeny. In a previous report, we compared the replicative cycles of these two viruses from the time of eclipse until cell death (21), attempting to identify temporal and morphological differences which might contribute to this disparity in replication times. In the present report we apply a similar approach to the early stages of viral penetration.

MATERIALS AND METHODS

Virus. The HF (8) strain of HSV and the AD-169 (25) strain of CMV were grown on monolayers of WI-38 cells as described previously (20). High multiplicities of infection (200 to 500 PFU/cell) were used in order to visualize a significant number of infecting particles by electron microscopy. To obtain these high titer inocula, clarified homogenates of infected cells were filtered through membrane filters (0.45 μm pore size; Millipore Corp.) and then concentrated either by centrifugation for 2 h at 20,000 $\times$ *g*, followed by resuspension in serum-free minimal essential medium (MEM); or by negative pressure ultrafiltration through dialysis tubing (20). Portions of all viral inocula, both HSV and CMV, were pelleted by centrifugation at 30,000 $\times$ *g* for 90 min into conical BEEM capsules held in adaptors for the SW-27.1 rotor. The pellets were examined by electron micros-

copy to determine the degree of morphological preservation of the viruses used in the penetration studies.

Cells. WI-38 cells in the 23rd passage were seeded into 35-mm plastic petri dishes (2×10^5 cells per dish) and incubated in MEM plus 10% fetal calf serum, at 37 C in 5% CO_2.

Infection of cells and sampling. Pre-confluent monolayers were chilled for 30 minutes at 4 C and then inoculated with 0.1 ml of chilled virus suspension at an input multiplicity of approximately 200 to 500 PFU/cell. Controls received 0.1 ml of a comparable preparation from uninfected cells. Adsorption was for 90 minutes at 4 C. One infected sample and one control were taken at the end of the adsorption period. The remaining plates were washed once with cold medium and then rapidly warmed to 37 C by the addition of pre-warmed MEM plus 2% agamma calf serum. Samples were taken for electron microscopy at intervals from 3 to 180 minutes after warming, with additional later samples up to 24 hours (HSV) or 96 hours (CMV).

Electron microscopy. Monolayers were fixed and embedded in situ as described previously (21). Thin sections were prepared with a Porter-Blum Ultratome, stained with uranyl acetate and lead citrate, carbon-coated and examined in a modified Siemens Elmiksop 1A (the main modification was a shorter objective focal length; H. Armbruster, Siemens, Inc., Islein, N.J.) using a double condenser, a liquid nitrogen-cooled anti-contamination device, and an accelerating voltage of 80 kV.

RESULTS

Thin sections of pelleted viral inocula, when examined by electron microscopy, showed that for both HSV and CMV the morphology of most particles was comparable to that of normal viruses within infected cells (21). Physical damage seemed limited to occasional discontinuities in the envelope, and was not widespread. Contamination of HSV samples by nonviral material appeared minimal, regardless of the method of inoculum preparation. CMV samples contained relatively more membranous debris, presumably due to the necessity of using larger numbers of cells to recover equal numbers of plaque-forming units. The CMV preparations also contained membrane-bounded homogeneous dense bodies which will be described below.

Cells exposed to virus and harvested at either 24 (HSV) or 96 h (CMV) clearly showed the replication of virus in virtually all cells, thus insuring that productive infection had occurred as a result of exposure to these inocula.

Monolayers exposed to either virus and then sampled at 0 min, i.e., without warming, showed numerous adsorbed viruses, but only rarely showed images of other viral-cell interactions, indicating that, as previously reported (7), adsorption at low temperature was effective in inhibiting viral penetration. From a comparison of the percentage of enveloped particles in an HSV inoculum prior to adsorption (69.0%) versus the percentage of adsorbed particles with envelopes (96.6%), it seemed that enveloped viruses were preferentially adsorbed to the cells. The same appeared to be true for CMV. Occasionally the gap between the viral envelope and the plasmalemma appeared to be filled by an amorphous layer of unknown material (Fig. 1b). More often no visible link existed between the two.

In samples taken 3 min after warming, two types of virus-cell interaction were evident, namely phagocytosis of virus and fusion between the plasmalemma and the viral envelope. Clear images of fusion were observed with both HSV and CMV. These images may be presented in a sequence which seems likely to illustrate the order in which the observed events probably occur during penetration (Fig. 1, 2). Fusion appeared to begin at one or more small foci of contact between the viral envelope and the plasmalemma, and to proceed very rapidly, since naked viral capsids free in the cytoplasm were already observed in the 3-min samples.

The viral envelope appeared to be intercalated into the plasmalemma as a result of this fusion. In certain cases it appeared possible to postulate the location of the former envelope within the plasmalemma even without the use of any markers. Figure 1 d shows an HSV capsid just beneath the surface of the cell, and between the two an aggregate of granular electron-dense material identical to that often found (21) between the capsid and the envelope of herpesviruses. Since this material apparently diffuses away rapidly into the cytoplasm, its presence strongly suggests that the membrane protrusion in Fig. 1d is a viral envelope which has recently fused with the plasmalemma.

Discontinuities in apposed regions of the viral envelope and the plasmalemma were rarely encountered and did not seem to play any role in fusion. Those breaks which were observed in the envelopes of adsorbed viruses occurred with no greater frequency than in the inoculum. Furthermore, they were not confined to the region of apposition between viral envelope and plasmalemma, but rather occurred randomly over the entire surface of the adsorbed particle (Fig. 1b).

In the 3-min samples from either virus, phagocytosis of particles, either singly or in groups, occurred. Particles of all morphologies, enveloped and unenveloped alike, were phagocytosed and could be found in the cytoplasm within vacuoles of two distinct types. Most of those in the deeper areas of the cytoplasm,

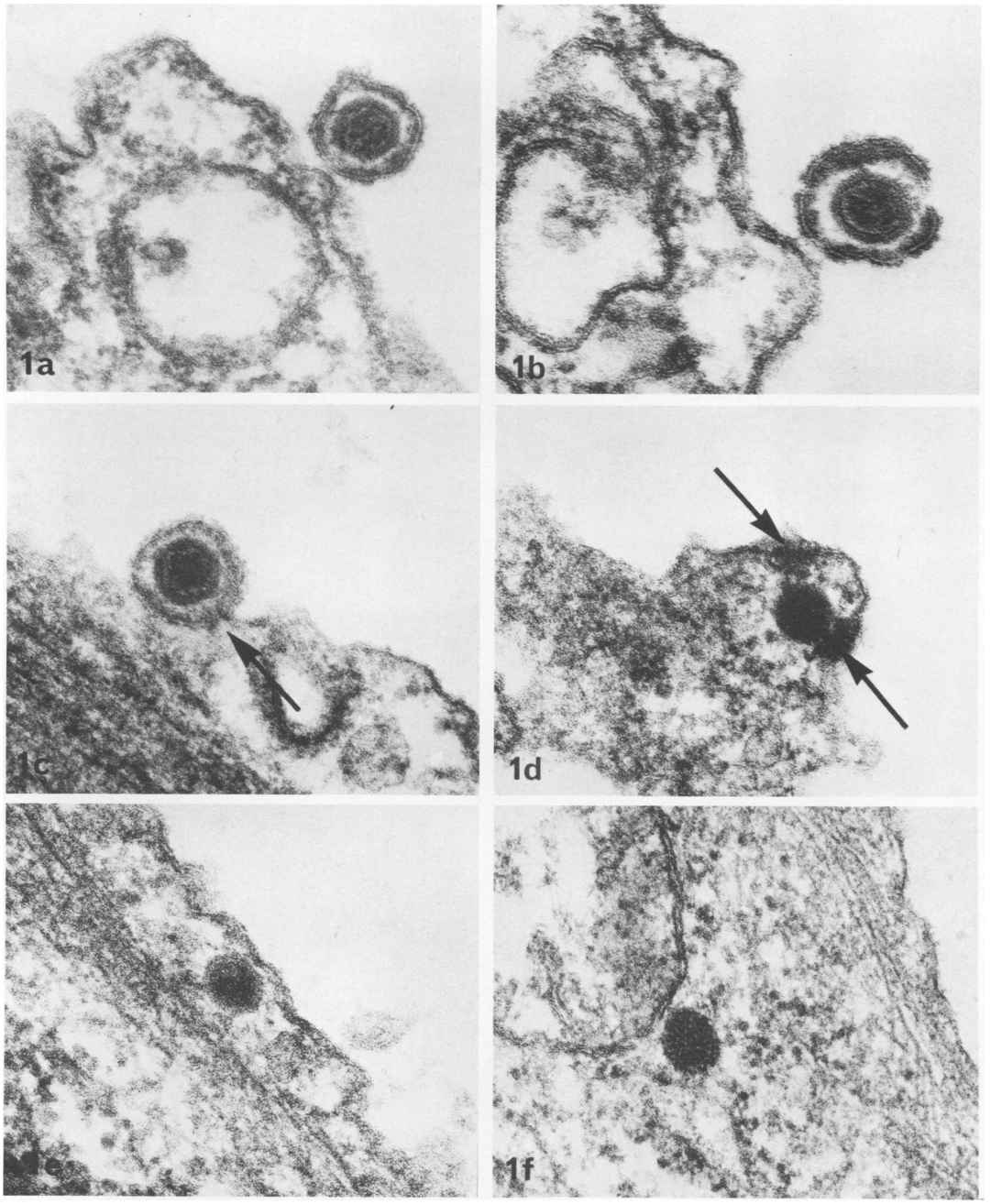

FIG. 1. *Stages in the penetration of HSV. (a) Adsorbed particle with intact viral envelope closely apposed to intact cell membrane; 15 min postwarming. Magnification ×90,000. (b) Amorphous material situated between a viral envelope and the plasmalemma; 15 min postwarming. Magnification ×95,000. (c) Viral envelope continuous with plasmalemma via a narrow isthmus (arrow). To the left of the fused particle, the plasmalemma is indistinct due to tangential sectioning through the in situ embedded specimen; 3 min postwarming. Magnification ×90,000. (d) Naked capsid free in the cytoplasm. Note the patches of electron-dense material (arrows) between the capsid and the surrounding membrane. This material is indistinguishable from that often found between the envelope and capsid of HSV. Its presence suggests that the adjacent membrane was originally a viral envelope, 3 min postwarming. Magnification ×80,000. (e) A naked capsid immediately beneath the plasmalemma. This virus has probably penetrated into the cytoplasm very recently, since it still remains the regular and well-defined structure typical of extracellular herpesviruses; 5 min postwarming. Magnification ×80,000. (f) A typical rounded capsid with no coat, located deeper in the ctyoplasm; 5 min postwarming. Magnification ×90,000.*

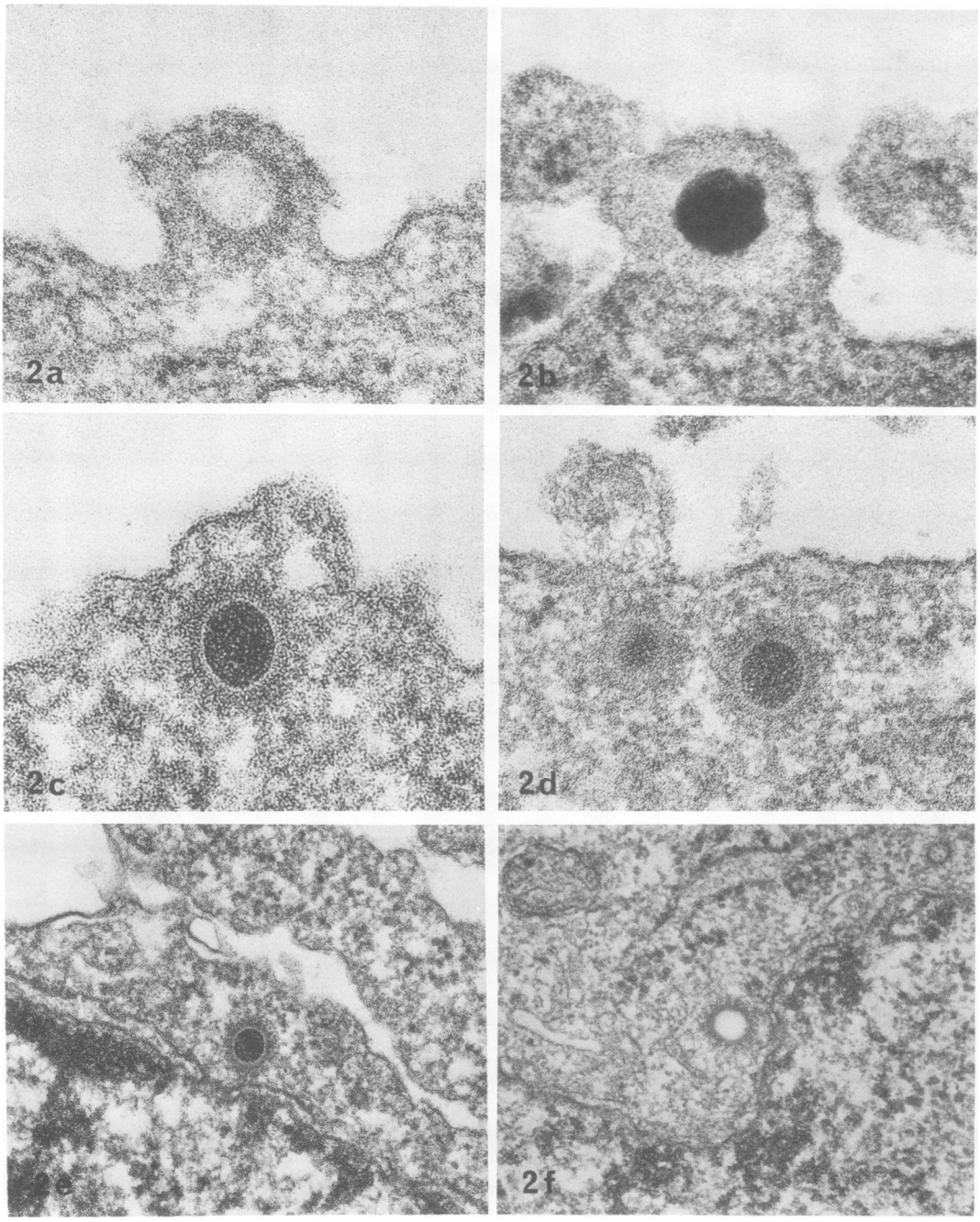

FIG. 2. *Stages in membrane fusion of CMV. Some variation occurs in the timing of coating. Thus, although most capsids in the early morphological stages of fusion are not yet coated (b), an occasional capsid appears to become coated almost immediately upon contact with the cytoplasm (a). Virtually all naked capsids located deeper in the cytoplasm are coated (c–f). Capsids of all morphologies, even those which appear "empty," become coated (a and f). Capsids are frequently encountered in the vicinity of a nuclear pore (e and f). (a–c) Five minutes postwarming, magnification approximately ×135,000; (d) 30 min postwarming, magnification ×120,000; (e–f) 30 min postwarming, magnification ×58,000.*

especially in the Golgi region, had a dense background matrix, and contained membranous fragments and other debris in addition to viruses (Fig. 3a and b). The viruses within the dense vacuoles were often unenveloped and appeared slightly degraded, suggesting that these structures represented phagolysosomes where viral breakdown was occurring. Most vacuoles nearer the surface of the cell, implying a more recent formation, had a clear matrix, and usually contained nothing other than viral particles (Fig. 3c). In the clear vacuoles, most viral particles appeared intact, suggesting that these structures were phagosomes which had not yet fused with vesicles carrying lysosomal enzymes.

No recognizable viral components were ever observed escaping from a phagolysosome-like vacuole. However, the capsids of enveloped virions did appear to be released from clear phagosome-like vacuoles as a result of fusion between the viral envelope and the vacuolar membrane. This process was analogous to fusion with the plasmalemma. The envelope of a single virus particle in a small vacuole sometimes fused with the phagosomal membrane at several loci (Fig. 3c). Figure 3d represents a likely later stage in this same process. In other cases viral envelopes fused with the wall of larger vacuoles at a single point, producing images such as that in Fig. 3e.

Three major distinctions were noted between HSV and CMV penetration. First, the naked capsids of CMV, once free in the cytoplasm, were almost always coated by a fine fibrillar material arranged in a radial or spoke-like pattern (Fig. 2a and c–f, 3f, 5, and 6a). The exceptions were those very close to the plasmalemma or the wall of a phagosome, implying recent entry into the cytoplasm (Fig. 3e). Close examination of the original CMV inoculum showed no distinct coats on enveloped virions prior to penetration. HSV capsids free in the cytoplasm were never coated (Fig. 1d–f).

Second, the coated capsids of CMV, once free in the cytoplasm, retained their characteristic hexagonal shape in cross section for up to 1 to 1.5 days, whereas the uncoated capsids of HSV took on a somewhat rounded, less distinctly defined outline soon after entering the cytoplasm (Fig. 1f).

Finally, whereas the great majority of the HSV particles were no longer recognizable by electron microscopy at 2 h after infection, CMV particles did not reach a comparable stage of eclipse until 1.5 days postinfection. For CMV, the long period of time elapsing before viral eclipse meant that the particles accumulated in fairly large numbers within the cell, probably with negligible losses of physical particles during the early minutes after penetration. This made it possible to determine the distribution of viral particles within the cells as a function of time. In each sample, 200 virions were counted and classified into the categories shown in Tables 1 and 2. By 5 min postwarming, more than half of the viral particles (55%) were observed within the cytoplasmic matrix as naked capsids, presumably after a membrane fusion process (Table 1). Phagocytosis also occurred within the first 5 min, but was less frequently observed than fusion. The frequency of phagocytosed particles increased slowly thereafter. Escape from phagosomes began between 5 and 30 min. The percentage of virions in the process of fusion (Table 2) was highest (18%) at the earliest sampling time and gradually decreased to undetectable levels by 90 min. These data suggest that fusion is a rapid process occurring almost immediately after warming.

Naked capsids free in the cytoplasmic matrix were originally most numerous just beneath the plasmalemma, as expected. But even at 5 min postwarming, an almost equal number had moved into deeper areas of the cytoplasm, and in fact some were already in the vicinity of the nucleus. There the majority were characteristically located near a nuclear pore (Fig. 2e and f). The percentage of capsids below the plasmalemma peaked at 30 min and then decreased, while larger numbers began to accumulate in the Golgi region (Fig. 3f) as well as in the vicinity of the nucleus. By 90 min many more naked capsids were located near the nucleus than at any other single cytoplasmic location. Observation of HSV samples also indicated rapid penetration of naked capsids to the nucleus and their accumulation at the nucleus and in the Golgi. No attempt was made to construct a time distribution for HSV because of the rapid eclipse of viral particles.

Despite frequent observation of HSV and CMV particles near the nucleus, no breakdown of these capsids, such as might accompany the release of the genome, could be seen. Furthermore there was no evidence of any interaction with the nuclear pores, nor of any alterations in the subadjacent nucleoplasm. Similarly, the ultimate fate of capsids in the Golgi region could not be immediately ascertained.

The picture which emerges of virus-cell interaction during penetration is summarized

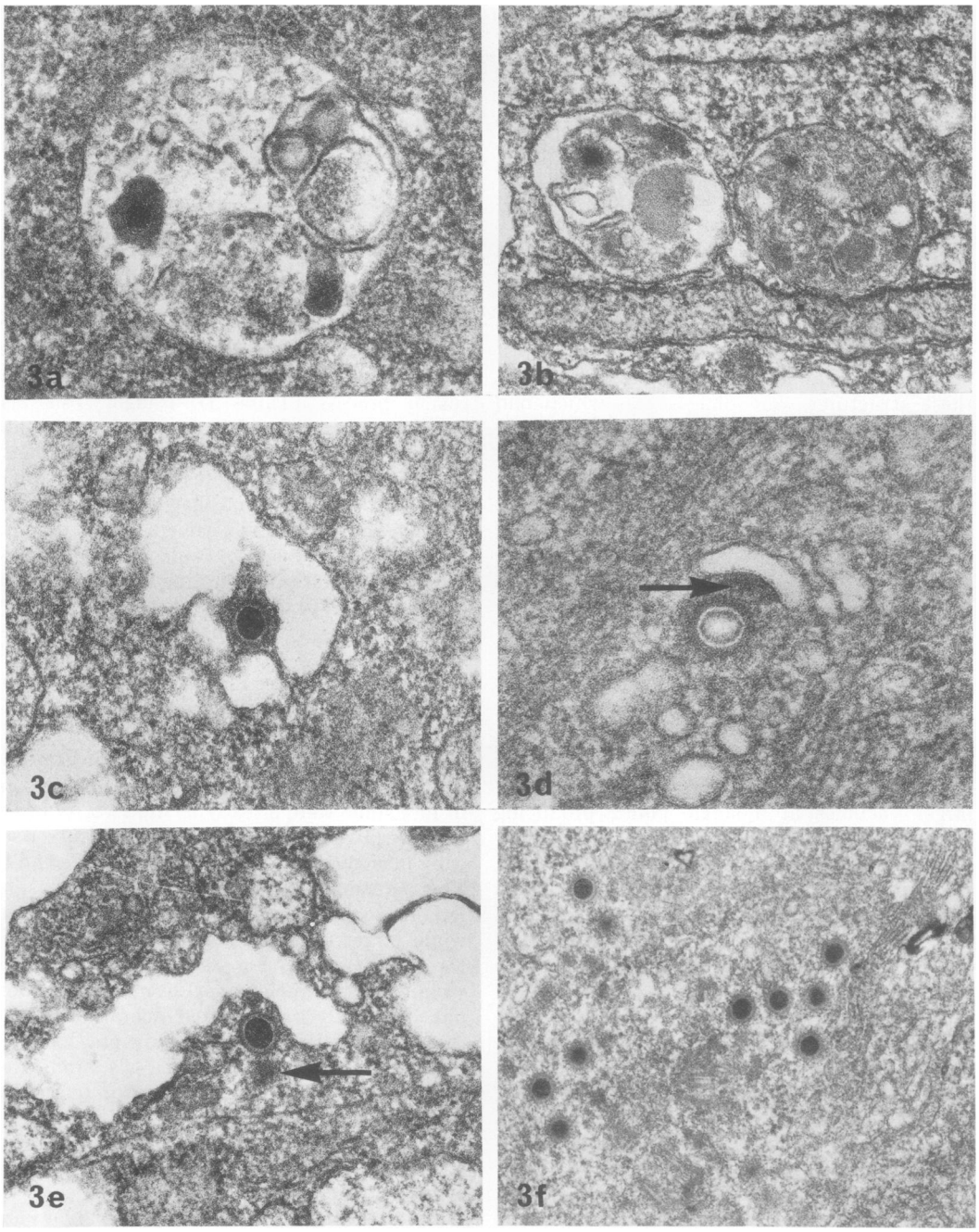

FIG. 3. (a) *One, or possibly more, HSV particles within a phagolysosome-like vacuole also containing debris; 60 min postwarming. Magnification* ×*45,000.* (b) *Disrupted CMV particles and several dense bodies within phagolysosome-like vacuoles similar to that in a; 30 min postwarming. Magnification* ×*40,000.* (c) *An enveloped CMV particle fusing at several loci with the membrane of a clear phagosome-like vacuole; 30 min postwarming. Magnification* ×*50,000.* (d) *A later stage of multiple fusion between an enveloped CMV particle and the membrane of a phagosome. The phagosome is now represented only as a number of small vesicles surrounding the capsid. Traces of electron-dense material remain (arrow) between the capsid and the probable former viral envelope; 5 min postwarming. Magnification* ×*85,000.* (e) *A naked CMV capsid which has apparently just entered the cytoplasm from the adjacent phagosome. The capsid still remains uncoated and the dense material between capsid and envelope has not yet entirely diffused away (arrow); 30 min postwarming. Magnification* ×*50,000.* (f) *A typical accumulation of coated CMV capsids in the Golgi region; 90 min postwarming. Magnification approximately* ×*35,000.*

TABLE 1. *Cellular distribution of CMV particles as a function of time*

Cell location	Time (min)			
	5	30	60	90
Cell surface	41.0[a]	8.5	3.0	2.0
Phagosomes	4.0	6.5	10.5	7.0
Cytoplasm	55.0	85.0	86.5	91.0
Below plasma membrane	29.0	33.5	16.5	10.5
Golgi region	0.0	6.5	18.0	15.5
Nuclear periphery	5.5	8.5	20.0	40.0
Other regions	20.5	36.5	32.0	25.0

[a] In percentage of the 200 viral particles counted for each sample time.

schematically in Fig. 4 for CMV. Many of the features presented diagramatically in Fig. 4 can be identified in the micrograph shown in Fig. 5. The available evidence indicates that a very similar course of events occurs in HSV penetration with the three exceptions noted above: lack of coating on HSV capsids, rapid loss of well-defined HSV capsid morphology, and rapid HSV disassembly.

As mentioned earlier, in addition to viral particles the typical inoculum of CMV (but not HSV) contained membrane-bounded bodies composed of a finely granular, homogeneous, electron-dense material. Their chemical nature is not clear at present. When added to uninfected cells as part of a CMV inoculum, these dense bodies were able to adsorb, were phagocytosed (Fig. 6a), and fused with the plasmalemma (Fig. 6b) in the same manner as virus. Since the electron-dense material comprising these bodies could not be identified within deeper regions of the cell, it is supposed that it diffuses rather quickly into the cytoplasm.

The early responses of the cells to infection with high input multiplicities of virus differed in several ways from the events observed with lower (5 to 10 PFU per cell) inputs (21). First, the higher multiplicity of either virus caused the production of more numerous and larger polykaryocytes (Fig. 6c). Second, polykaryocytosis occurred earlier in the infectious cycle, beginning within 5 to 15 min after the initiation of penetration. Third, there was more frequent and more extensive reduplication of the nuclear membrane (Fig. 6d), and fourth, this phenomenon also appeared at an earlier time, usually within the first 2 to 3 h after penetration by either HSV or CMV. These changes were especially noteworthy in the case of CMV, since this virus, when inoculated at lower multiplicities, produces only very limited polykaryocytosis or nuclear membrane reduplication, and only at considerably later times in the cycle (1 and 4 days postinfection, respectively) (21).

DISCUSSION

These results show that like HSV (4, 6, 9, 10, 13, 16, 17) and other members of the herpesvirus group (26), extracellular CMV initially interacts with the cell in one of two basic ways, either via phagocytosis, or fusion with the plasmalemma. The occurrence of fusion between CMV envelopes and the plasmalemma had been postulated earlier by Iwasaki et al. (12), who observed naked capsids free in the cytoplasm soon after infection.

The occurrence of both phagocytosis and fusion has been observed during the penetration of a number of viruses into susceptible hosts (3, 4, 10, 12, 13, 14, 15, 17, 18, 19, 26), raising the question of which process is actually responsible for the initiation of viral infection. Even though the definitive answer to this question is difficult if not impossible to obtain using morphological methods of investigation, nevertheless we would point out the following. First, according to our observations, no recognizable viral component ever reaches the cytoplasm, either from the extracellular space or from a phagosome, except by a fusion event. Second, the naked capsids which result from fusion are transported across the cytoplasm in a rapid and orderly manner, and localized near the nucleus. This sequence suggests, but does not establish, that such particles are involved in the initiation of viral replication, and therefore that penetration via membrane fusion is an important process in productive infection by herpesviruses. It seems significant in this respect that the only morphologically observable events involving phagocytosed virions were viral escape from the phagosome via membrane fusion or apparent viral degradation within lysozyme-like vacuoles. Release of viral components from phagosomes via a similar fusion mechanism has also been re-

TABLE 2. *Frequency of CMV particles in the process of fusion as a function of time*

Cell location	Time (min)			
	5	30	60	90
Fusing at cell surface	18.0[a]	8.0	5.0	0.0
Fusing at phagosomal membrane	0.0	6.5	2.5	0.0
Total	18.0	14.5	7.5	0.0

[a] In percentage of the 200 viral particles counted for each sample time.

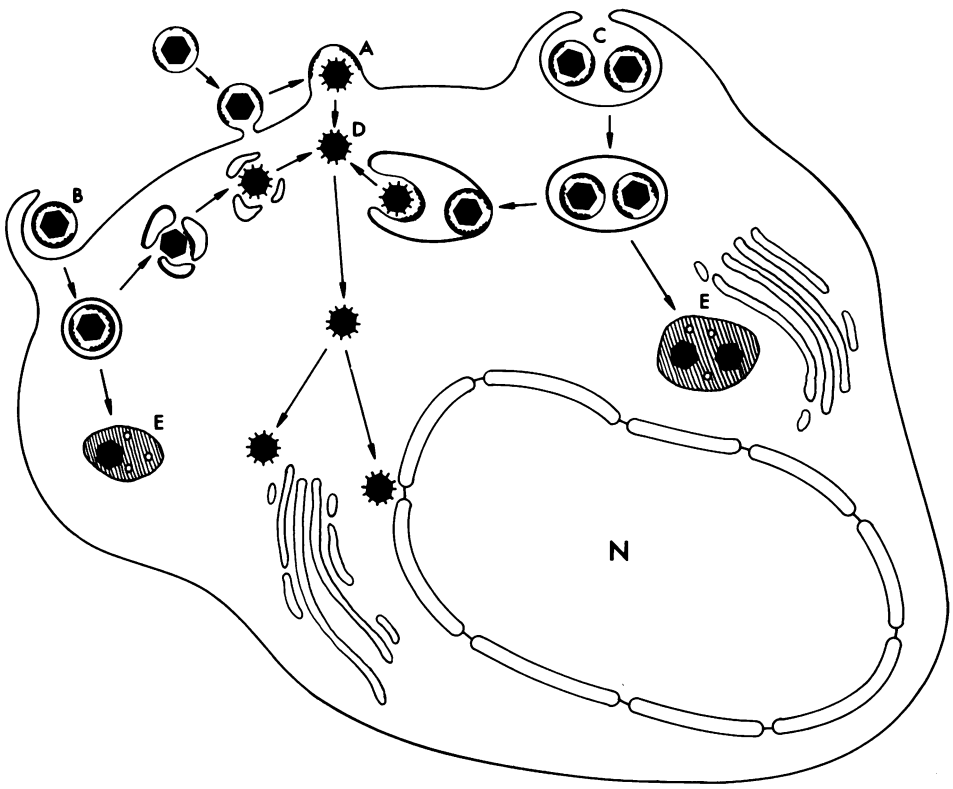

FIG. 4. *Diagramatic representation of the interaction of CMV with the cell in the early stages of infection. Enveloped particles at the cell surface either fuse with the plasmalemma (A), or are taken up singly (B) or in groups (C) into phagosomes. Naked particles appear to follow only the phagocytic routes. Within the phagosomes the enveloped particles may fuse with the vacuolar membrane to release naked capsids into the cytoplasm. There they receive a coat (D) and are indistinguishable from the particles which fused at the cell surface. Alternatively, the particles may remain in the phagosomes until, after fusion with lysosomal elements, they become phagolysosomes (E). Such particles often lose their envelopes and may be completely digested within these structures. No release of capsids from phagolysosome-like vacuoles was ever observed. Naked capsids appear to be transported through the cytoplasm and tend to accumulate in either the Golgi or the perinuclear regions.*

ported with rabies virus (11), and has been suggested for both HSV (10) and CMV (12).

Arrival of both HSV and CMV capsids at the nucleus within 5 min after warming correlates well with the autoradiographic data of Hummeler et al. (10) who used as their inoculum HSV labeled with [^{3}H]thymidine, and first observed silver grains over the nucleus between 15 and 30 min postinfection.

It is interesting to note that up to this point, i.e., the arrival of capsids at the nucleus, the time course for penetration by these two viruses is indistinguishable. However the times necessary for disassembly of visible viral particles differ greatly for the two, with HSV entering eclipse between 90 and 120 min postinfection, whereas CMV particles remain for 1 to 1.5 days.

It is not clear how the transport of particles to the nucleus was effected. The role of microtubules in the process, which has been postulated in the case of adenoviruses (2), could not be assessed here, because these structures are not well preserved under the conditions of fixation utilized.

The observation of naked cytoplasmic capsids near a nuclear pore was a constant one. This is especially true in the case of CMV, where large numbers of particles were seen near the nucleus. Although no further interaction of capsid and nuclear pore were observed by us, it is possible that a specific relationship does exist between the two. A similar association between the capsids of input virions and nuclear pores has been reported for several adenovirus types (1, 2). Also, the pores are structures which have frequently been implicated in the exchange of

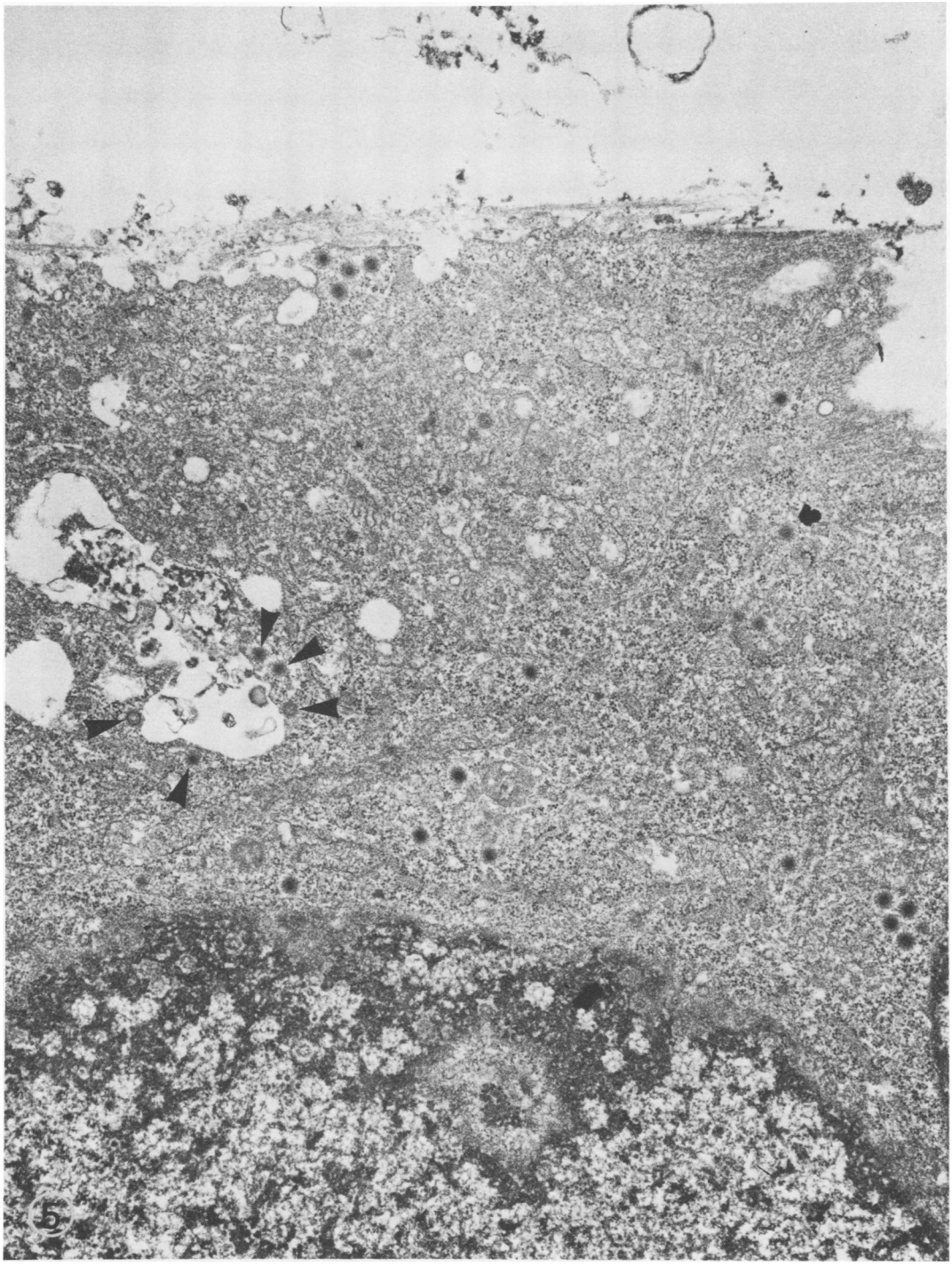

FIG. 5. *Numerous coated CMV capsids in the cytoplasm of a cell 90 min after the initiation of penetration. A group of capsids on the left (arrowheads) surrounds the periphery of a phagocytic vacuole, suggesting recent fusion of their envelopes with the vacuolar membrane. Other capsids are found just beneath the plasmalemma, at deeper locations in the cytoplasm, and in the vicinity of the nucleus. Magnification approximately ×20,000.*

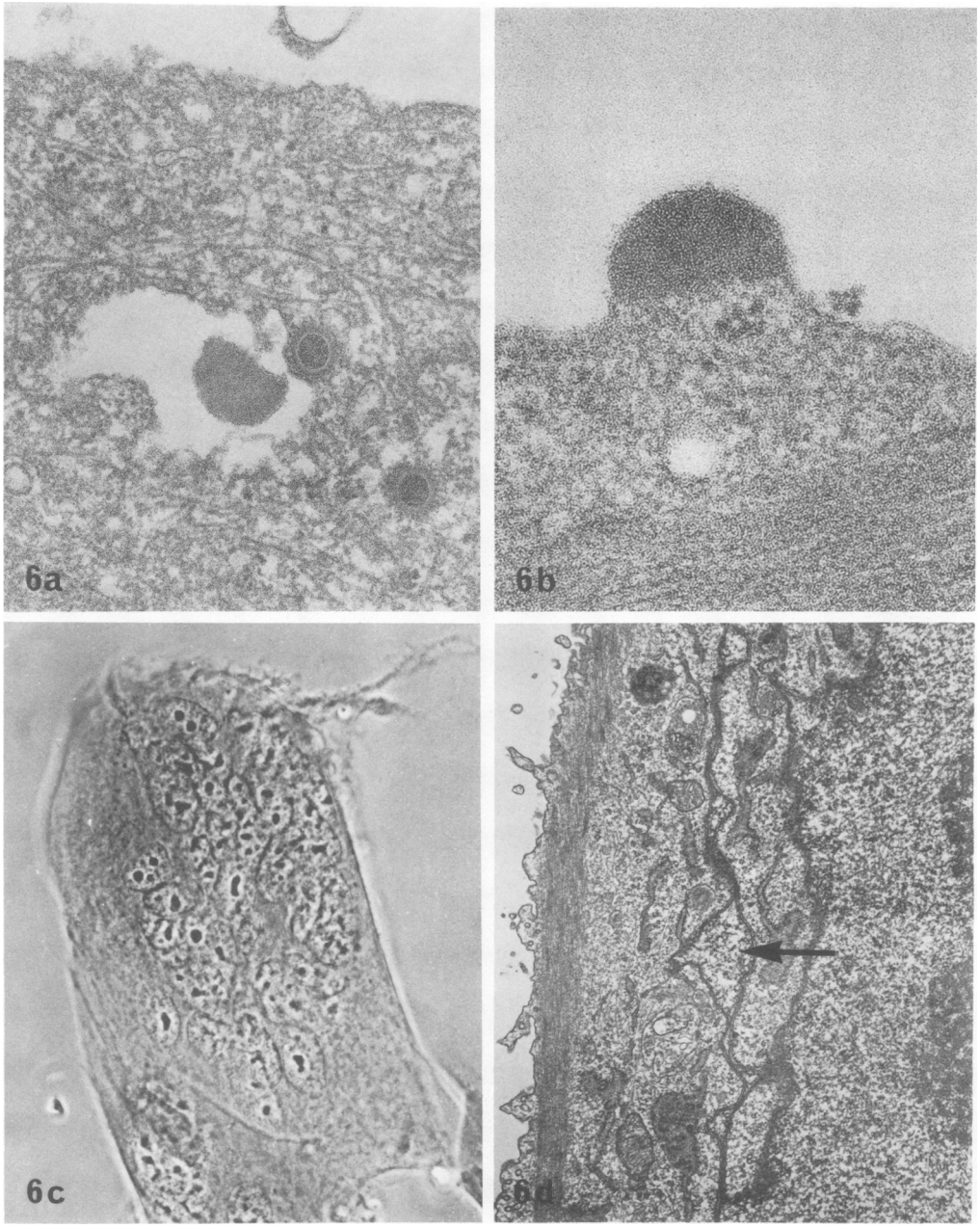

FIG. 6. (a) *A dense body within a phagosome 60-min postwarming. Several coated CMV capsids are already free in the cytoplasm. Magnification* ×*65,000.* (b) *A dense body, still retaining its contents, which has fused with the plasmalemma; 5 min postwarming. Magnification* ×*120,000.* (c) *A polykaryocyte produced by infection with high multiplicities of CMV. Cell fusion in response to such inocula began within 5 min of the initiation of penetration; 12 h postwarming. Magnification* ×*400.* (d) *Reduplication of the nuclear membrane and looping out of the membrane into the cytoplasm 12 h after infection by high multiplicities of CMV. The elaboration of the membranes, which carried small pockets of nuclear material deep into the cytoplasm (arrow), was far more extensive and appeared days earlier than that seen in infection by lower viral multiplicities (21). Magnification* ×*10,000.*

materials between the cytoplasm and the nucleus (23).

The most striking morphological difference between HSV and CMV penetration was the fact that the capsids of CMV became coated with a fibrillar material after entry into the cytoplasm, whereas those of HSV did not. These results support our earlier conclusion (21) that at least in this system the nature of the virus and not of the host cell determines the occurrence of coating. The fact that this coating occurred within minutes after viral penetration into uninfected cells implies that the coat material is not a product of virus-directed synthesis, but, rather, exists within the cytoplasm prior to infection. The possibility exists that this coat may be related to, or indeed may be responsible for, the prolonged maintenance of well-defined capsid structure in naked cytoplasmic CMV, and for the slow disassembly of this virus.

Coats have also been reported on naked cytoplasmic capsids of Epstein-Barr virus (5, 24), Lucké virus (22), and human CMV (21), all late in productive replicative cycles. In these cases the particles involved probably represent newly formed progeny virus rather than the parental or input virus. Such coats appear to become morphologically disorganized or lost if such capsids are subsequently enveloped (21). Other herpesviruses such as HSV show no coating of either progeny (21) or penetrating parental capsids free in the cytoplasm.

This report is, to our knowledge, the first to demonstrate the coating of a penetrating herpesvirus. Subsequent experiments are planned to elucidate the effect of coating on the course of infection, especially the possible relationship between coating and slow viral disassembly. In this connection, it seems significant that the naked capsids of HSV, which are not coated after penetration, rapidly become rounded or irregular in outline, suggesting that they may be undergoing disassembly. A similar observation has been reported for HSV entering HeLa cells (13).

The ability of the dense bodies found in CMV inocula to adsorb to and fuse with cell membranes in analogy with the behavior of virus, probably reflects a similarity between their limiting membranes. Both virus and dense bodies are frequently enveloped in the Golgi region of infected cells, by morphologically similar mechanisms, and during the same period of the infectious cycle (21). Along with the fact that enveloped particles of all types, even those with empty capsids, are able to fuse with the plasmalemma, the behavior of dense bodies suggests that fusion is a function of a particle's limiting membrane and not of its contents. The nature of these dense bodies and their importance, if any, in the infectious cycle of CMV remain unclear.

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