

## Putative Site for the Acquisition of Human Herpesvirus 6 Virion Tegument

EHUD ROFFMAN,<sup>1</sup> JOHN P. ALBERT,<sup>2</sup> JULIE P. GOFF,<sup>2</sup> AND NIZA FRENKEL<sup>1\*</sup>

*Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases/Twinbrook, 12441 Parklawn Drive,<sup>1</sup>  
and Division of Molecular Virology and Immunology, Georgetown University Medical Center,<sup>2</sup>  
Rockville, Maryland 20852*

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**The virion of human herpesvirus 6 (HHV-6) contains a very distinct tegument layer, occupying the space between the nucleocapsid and the virion envelope. Ultrastructural analyses of thymocytes infected with HHV-6 revealed the presence of intranuclear spherical compartments, approximately 1.5  $\mu\text{m}$  in diameter, in which tegumentation seems to take place. These compartments, termed tegusomes, were bounded by two membranes and contained ribosomes, consistent with their derivation by cytoplasmic invagination into the nucleus. Capsids located within the nucleus outside the tegusomes were all naked, while those located in the cytoplasm were uniformly tegumented. In contrast, capsids present inside the tegusomes contained teguments of variable thicknesses. In addition, nucleocapsids were documented in the process of budding into the tegusomes. We thus suggest that the tegusomes represent a cellular site in which HHV-6 virions acquire their tegument.**

The recently identified T-lymphotropic human herpesvirus 6 (HHV-6) (4, 9, 15, 18) is the causative agent of exanthem subitum (17, 21), a prevalent childhood disease characterized by high fever and rash. Human herpesvirus 7 (HHV-7) is a new lymphtropic virus which was first isolated in our laboratory from CD4<sup>+</sup> T cells of a healthy individual (6). It replicates in fresh peripheral and cord blood lymphocytes, although its tropism has yet to be determined.

We have examined by transmission electron microscopy the structural viral intermediates seen in thin sections of cells infected with HHV-6 and HHV-7. We have noted the uniform and complete tegumentation of cytoplasmic capsids. The same observation was previously made for HHV-6 by Biberfeld et al. (1) and by Yoshida et al. (22). The term tegument was introduced by Roizman and Furlong (14) to describe the amorphous material occupying the space between the DNA-containing capsid and the envelope in the herpes simplex virus (HSV) virion. By comparison with HSV, the lymphtropic herpesviruses HHV-6 and HHV-7 contain a more prominent tegument. Moreover, the replicative cycles of HHV-6 and HHV-7 appear to be slower than that of HSV. These unique features facilitated studies concerning the cellular site for tegument formation. We report that HHV-6-infected thymocytes contain spherical, double-membrane intranuclear inclusions in which partially tegumented capsids are observed. The data suggest that naked nucleocapsids cross the two membranes of this structure. Capsids in the inner lumen of the structure seem to acquire the tegument before being released into the cytoplasm. Tegumented capsids in the cytoplasm were observed budding into cytoplasmic vacuoles, acquiring the virion envelope. Mature virions appear to be released from the cells by reverse endocytosis at the cell surface. A model depicting the proposed egress steps is shown (see Fig. 5). The intermediates presented are from HHV-6-infected human thymocytes. However, ultrastructural observations of peripheral blood lymphocytes infected with HHV-6 and HHV-7 showed similar results.

A culture enriched for mature thymocytes was prepared by incubation of fresh human thymocytes with peanut agglutinin, a lectin which agglutinates predominantly immature cells (11). The mature thymocytes recovered after removal of the agglutinated cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum, 0.1 U of recombinant interleukin 2 (Amgen Biologicals) per ml, and 1  $\mu\text{g}$  of phytohemagglutinin (Wellcome Diagnostics) per ml. On the following day the cells were infected with 0.001 50% tissue culture infective dose per cell of a cell-free preparation of HHV-6 strain Z29 (8) prepared in peripheral blood lymphocytes as previously described (3). On day 6 postinfection, at which time a moderate cytopathic effect was visible in light microscopy, the cultures were processed for transmission electron microscopy. Briefly, cell pellets were treated sequentially with glutaraldehyde and osmium tetroxide (1% each for 30 min in 0.1 M cacodylate buffer [pH 7.2]), stained with 1% uranyl acetate, and dehydrated in ethanol (30 to 100%). After propylene oxide treatment the samples were embedded in PolyBed 812 (Polysciences) and sectioned with an ultramicrotome. Sections of 70 to 80 nm thickness were examined in the Philips-300 electron microscope operated at 60 kV.

Structures representing different stages of virion maturation were noticed in an estimated 60% of approximately 3,000 cell sections which were examined. Infected cells containing different proportions of nuclear, cytoplasmic, and extracellular structures were observed, indicating an asynchronous infection. The low multiplicity of infection appeared to be advantageous in allowing the detection of structural intermediates which might be associated with various stages of virion maturation and egress. The nucleus of the HHV-6-infected cell seen in Fig. 1 contains exclusively naked viral capsids. The cytoplasm exhibits tegumented capsids (arrows) or mature virions which are seen associated with cytoplasmic vacuoles (arrowheads). The extracellular space contains only mature virions.

We noted that approximately 5% of the infected cell sections also contained one or two spherical intranuclear membrane-defined structures, approximately 1.5  $\mu\text{m}$  in diameter, in which tegumented capsids were observed. The

\* Corresponding author.

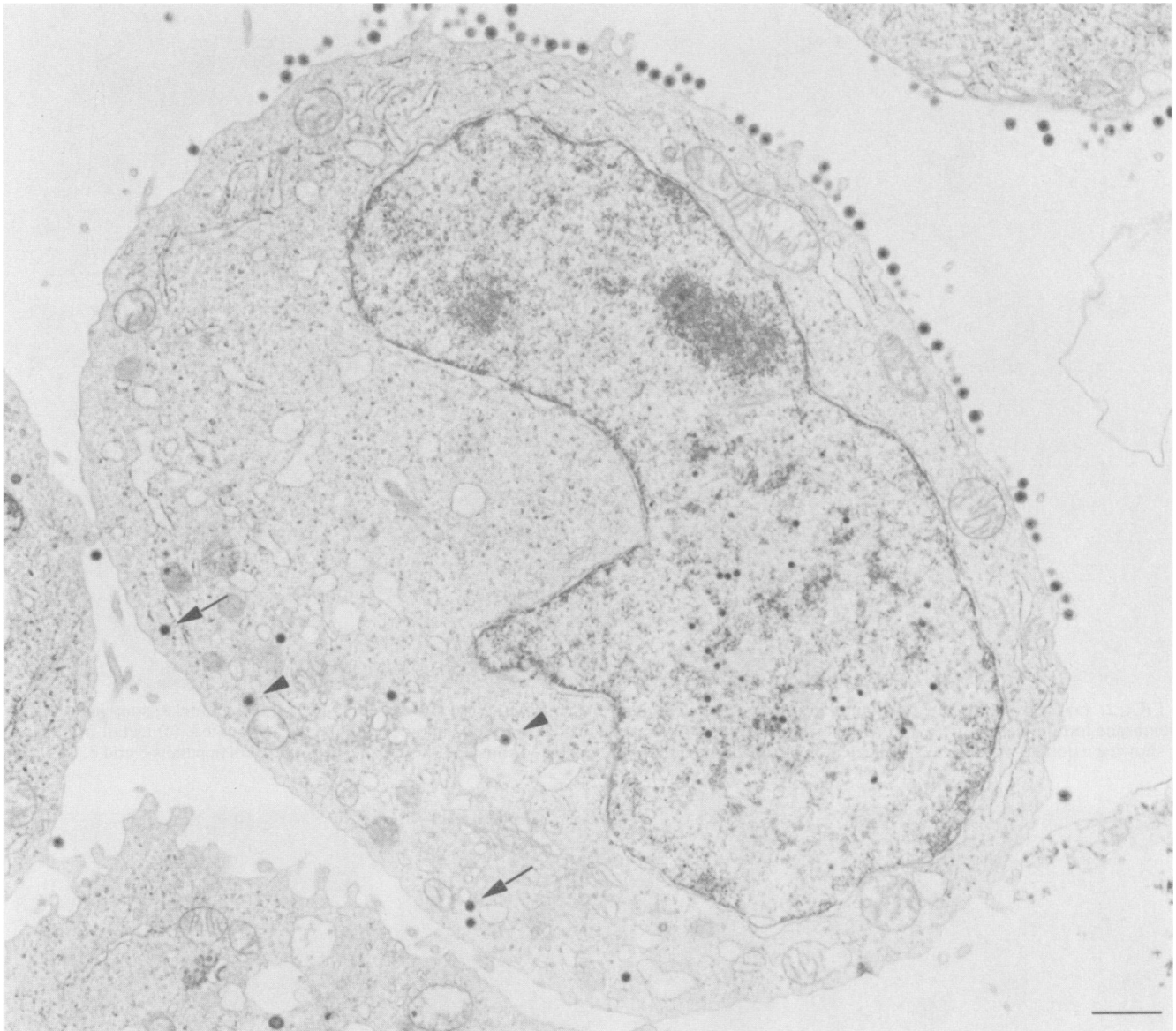


FIG. 1. HHV-6-infected cell exhibiting intracellular and extracellular viral structures. Note that nuclear capsids are naked, whereas cytoplasmic capsids are tegumented (arrows). Mature virions can be found in the cytoplasmic vacuoles (arrowheads) or outside the cell. Bar, 1  $\mu$ m.

cell shown in Fig. 2a contains two such intranuclear inclusions. One of these structures (enlarged in Fig. 2b) is composed of a single-membrane vesicle into which naked nucleocapsids with dense cores bud. During this process they acquire a membranous coat. The other structure (enlarged in Fig. 2c) is a double-membrane vesicle, in which two nonenveloped tegumented capsids are seen. We suggest that the intranuclear structures seen in Fig. 2 represent two different planes of sectioning of equivalent nuclear compartments.

This argument is based on structures such as those seen in Fig. 3a and b, in which nontegumented membrane-coated capsids (arrows) as well as tegumented capsids lacking the membrane coat (arrowheads) are seen together yet in different regions of the same intranuclear structure. The membrane-coated, nontegumented particles have a uniform

diameter of 145 nm and are found only within the intermembranal space of the structure discussed. However, as seen in Fig. 3c to e, the lumen within the inner membrane contains only nonenveloped capsids with variable thickness of tegument material. A plausible interpretation of these structures is that they represent capsids in the process of tegumentation. In Fig. 3c to e the arrows and arrowheads designate naked capsids and capsids with incomplete teguments, respectively. The naked capsids are typically 100 nm in size. Fully tegumented (nonenveloped) capsids in these lumens reach a size of 165 nm. The cytoplasmic origin of the observed nuclear structures is suggested by electron micrographs such as the one shown in Fig. 3f. In this figure the continuity of the inner lumen with the cytoplasm is demonstrated (arrowhead). In fact, the electron density of the inner lumen appears identical to that of the ribosome-rich cyto-

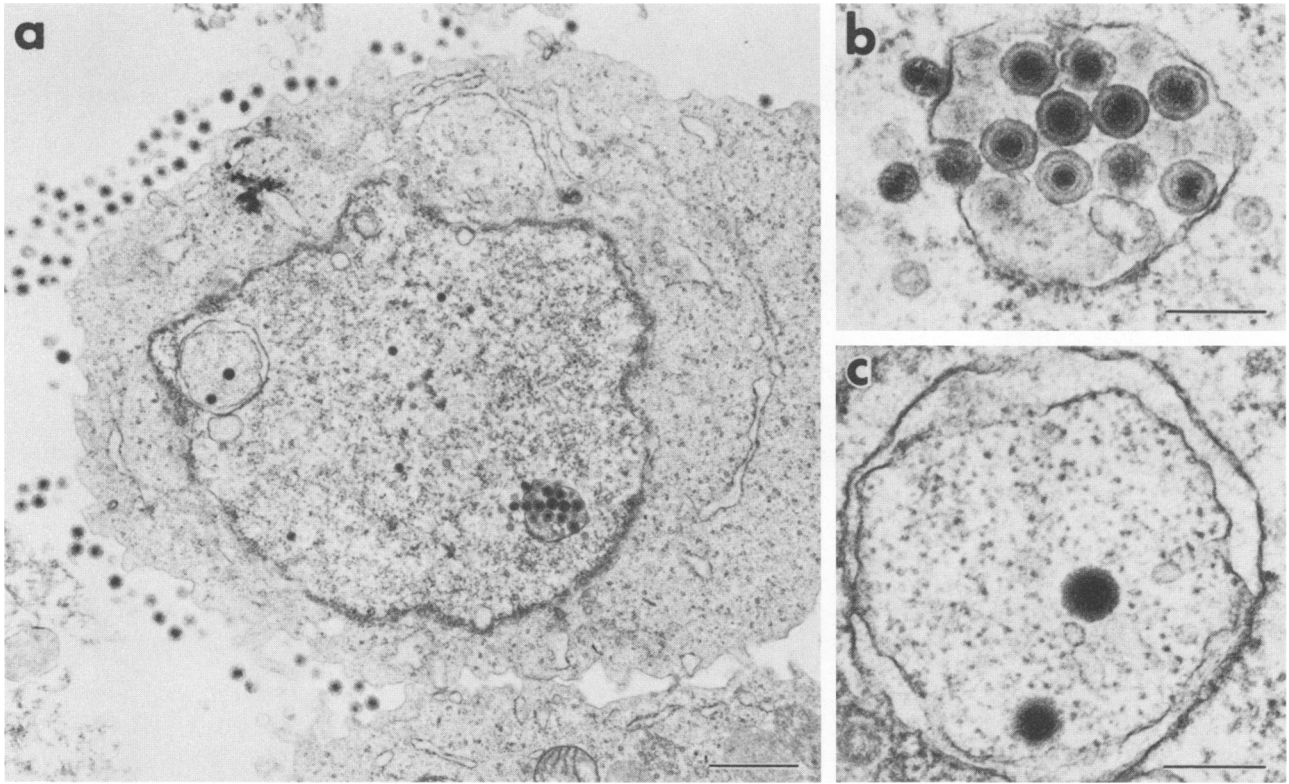
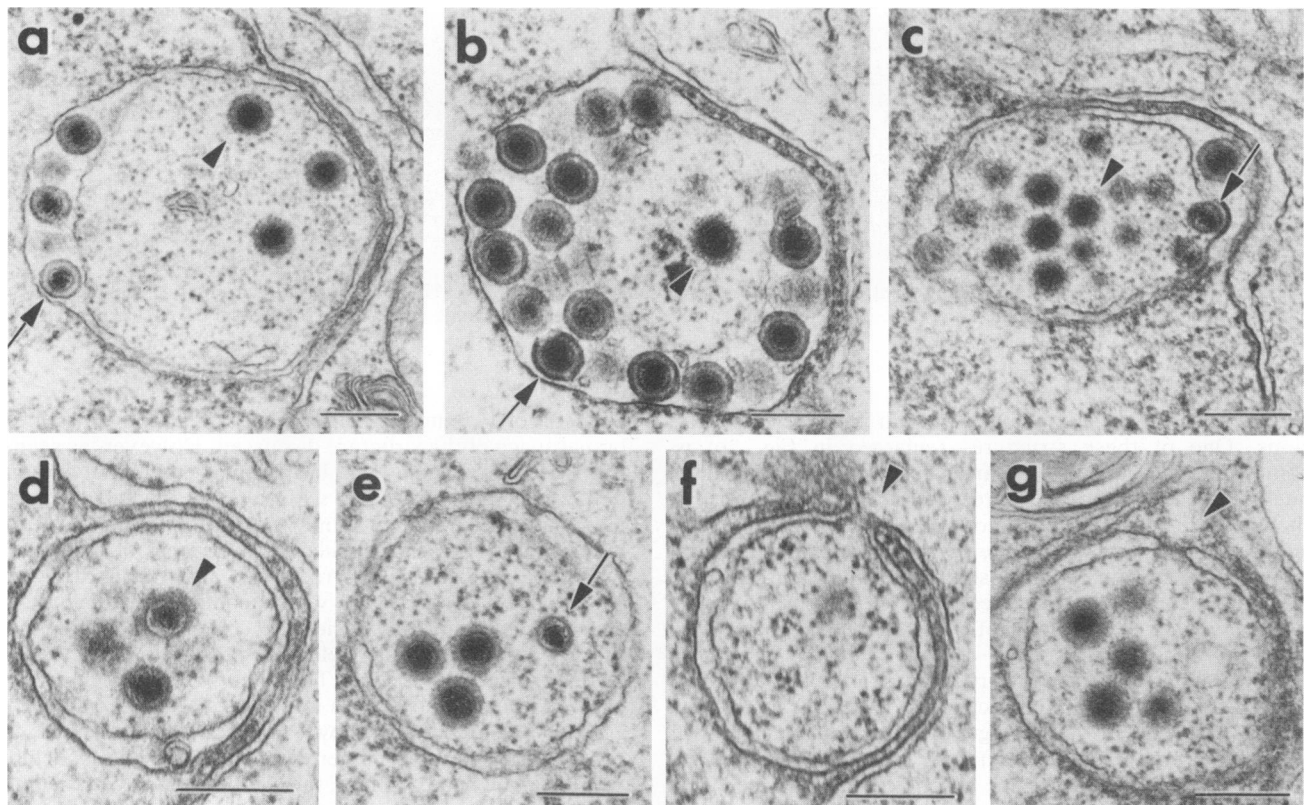


FIG. 2. (a) HHV-6-infected cell containing two intranuclear compartments with viral structures. (b) Detail from panel a, showing a single-membrane intranuclear vesicle into which naked capsids with high electron density bud and become membrane coated. (c) Detail from panel a, showing a double-membrane intranuclear inclusion containing two tegumented capsids. Bar in panel a, 1  $\mu\text{m}$ . Bars in panels b and c, 0.25  $\mu\text{m}$ .



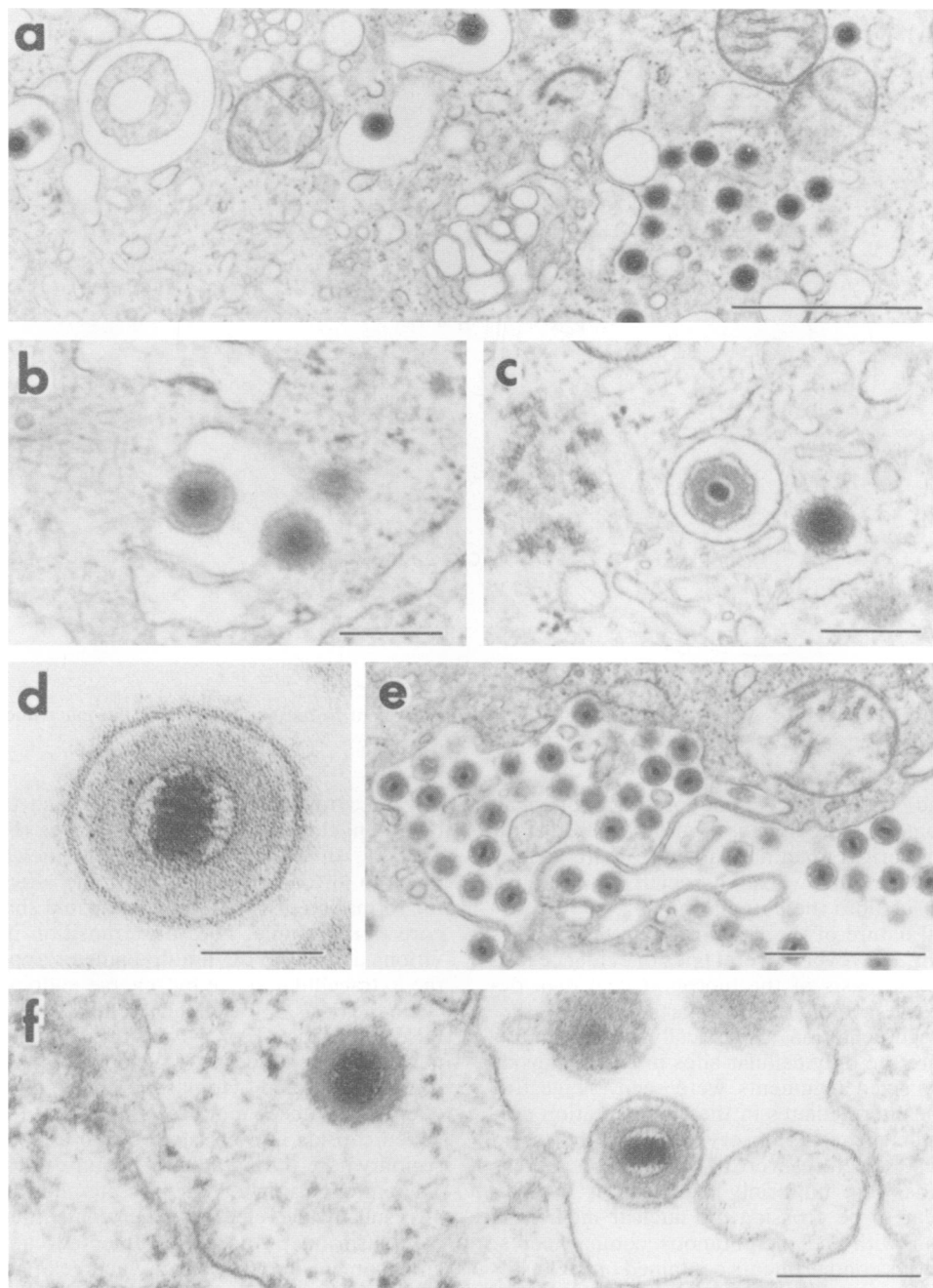


FIG. 4. Cytoplasmic and extracellular viral structures in HHV-6-infected cells. (a) Cytoplasmic segment containing tegumented capsids. (b) Tegumented capsid buds into a cytoplasmic vacuole and acquires the virion envelope. (c) Mature virion inside a cytoplasmic vacuole. (d) High magnification of an extracellular mature virion, exhibiting structural details. (e) Mature particles in cytoplasmic vacuoles, leaving the cell by reverse endocytosis. (f) Tegumented cytoplasmic capsid and an extracellular viral particle, demonstrating the change in the nucleoid structure occurring during virion maturation. Bars: panels a and e, 1  $\mu\text{m}$ ; panels b, c, and f, 0.25  $\mu\text{m}$ ; panel d, 0.1  $\mu\text{m}$ .

FIG. 3. Double-membrane intranuclear structures in HHV-6-infected cells. (a and b) Structures containing tegumented capsids in their inner lumens (arrowheads) and nontegumented membrane-coated capsids in the intermembranal region (arrows). (c to e) Structures containing naked capsids (arrows) as well as partially tegumented capsids (arrowheads). (f) Cytoplasmic invagination into the nucleus (the arrowhead points at a region of continuity with the cytoplasm). (g) Possible fusion event of membranes (arrowhead) at the cytoplasmic side of the intranuclear structure. Note that except for panel e, all the structures are positioned so that the nuclear side is to the left and the cytoplasmic side is to the right. Bars, 0.25  $\mu\text{m}$ .

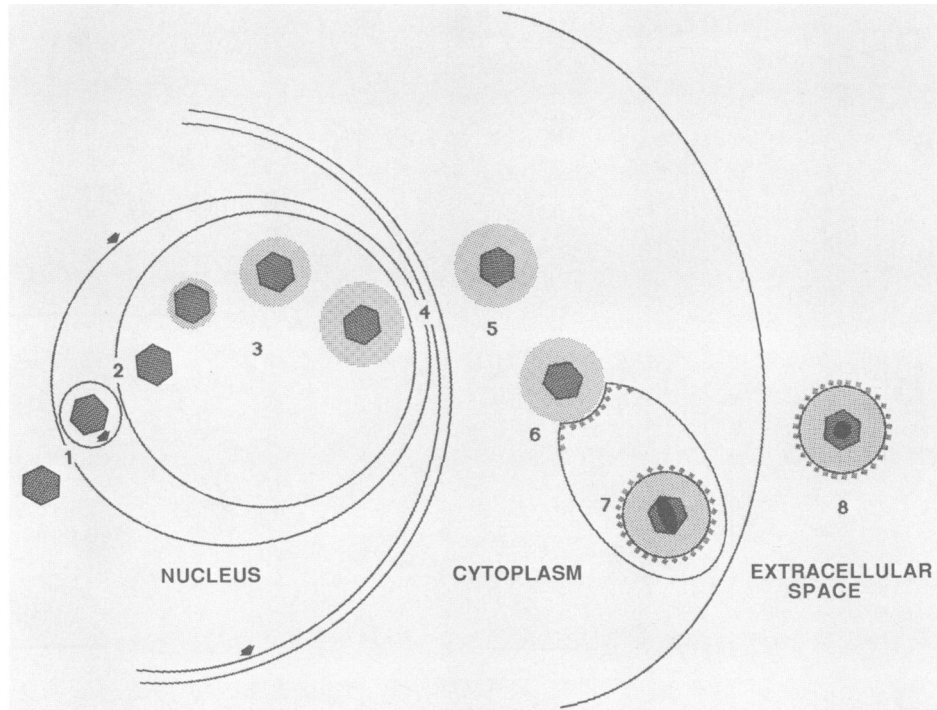


FIG. 5. Model for HHV-6 egress. Arrows point at membrane sides equivalent to the nuclear side of the inner nuclear membrane. For details see text.

plasm (Fig. 2 and 3). Moreover, the presence of small vacuoles within some of these lumens argues further in favor of their cytoplasmic origin (data not shown). Figure 3g may represent a fusion event (arrowhead) associated with transit of tegumented capsids into the cytoplasm.

The biochemical nature of the HHV-6 virion tegument is at present unknown. However, several lines of evidence lead us to suggest, on the basis of the morphological data described here, that the intranuclear compartments represent sites for capsid tegumentation. Specifically, the compartments described are the only cellular sites in which capsids with nonuniformly sized teguments were seen, suggesting that they represent intermediates in the tegumentation process. Moreover, nucleocapsids observed in the nucleus in regions outside these structures were always naked, whereas cytoplasmic capsids were uniformly tegumented. In addition, capsids appear to be crossing the nuclear membrane only in association with the membranous compartments. Last, the membranous compartments contained only capsids with electron-dense cores, presumably representing DNA cores, whereas empty capsids could be seen in the open nuclear zone outside these compartments (Fig. 2b). It thus appears that only full capsids find their way into the structures described. In additional studies (data not shown), nuclear inclusions such as the ones described here were not observed in mock-infected cells or in cells infected in the presence of high concentrations of interleukin 2 which restricted HHV-6 replication (12). It remains to be seen whether these structures, termed tegusomes, are unique to the lymphotropic HHV-6 and HHV-7 or whether they occur also in cells infected with other herpesviruses. The tegumentation process in cells infected with other herpesviruses might occur more rapidly, resulting in lower abundance of the partially tegumented intermediates.

The fully tegumented cytoplasmic capsids exhibit affinity

toward cytoplasmic vacuoles in which they appear to undergo envelopment and acquire spikes (Fig. 4a to d). Interestingly, during envelopment, the newly formed virions undergo an additional morphogenic alteration, resulting in the formation of an elongated ellipsoid shape of the nucleoid core (for example, note this transition in Fig. 4f). Mature virions averaging 185 nm in diameter appear to emerge into the extracellular space by reverse endocytosis (Fig. 4e).

On the basis of the data presented, we propose a model for HHV-6 egress (Fig. 5). In this model, DNA-containing nucleocapsids undergo envelopment at a membrane surrounding a sealed cytoplasmic invagination into the nucleus (step 1). A subsequent de-envelopment (step 2) releases the naked capsids into an islet of cytoplasm within the nuclear region where they gradually acquire full tegument (step 3). Thereafter, fusion events with the nuclear membranes (step 4) result in the release of the tegumented capsids into the cytoplasm (step 5). The tegumented capsids then undergo envelopment in cytoplasmic vacuoles (step 6), yielding mature virions (step 7). Fusion of the vacuole membrane with the cell membrane then releases the completed particles into the extracellular space (step 8). It is interesting that tegumented capsids were not observed in the intermembranal region of the nuclear structure, in support of the hypothesis that the nuclear structures are instrumental for the transfer of the tegumented capsids from the nucleus into the cytoplasm. It is also unlikely that the observed nuclear tegusomes serve to degrade viral structures, since damaged capsids were not observed therein.

Intranuclear vesicular structures were found in cells infected with other herpesviruses, including the Lucké frog renal adenocarcinoma virus (16), cytomegalovirus (5, 7), HSV (reviewed in references 2, 10, and 13), and herpesvirus saimiri (19). Yet, to the best of our knowledge, no such structures have been implicated with the acquisition of

tegument. It is noteworthy that we have previously shown for HSV that only capsids containing DNA molecules close to full-length standard virus DNA (but not smaller-sized defective virus genomes) are transported from the nucleus into the cytoplasm (20; B. Lum and N. Frenkel, unpublished data). A plausible explanation for this phenomenon could be that capsids containing full-length genomes are modified (e.g., by the addition of a maturation protein) so as to adhere to a tegusome equivalent, allowing tegumentation and further egress steps.

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