Herpesvirus Envelopment¹

ROBERT W. DARLINGTON AND L. HOWARD MOSS III²

Laboratory of Virology, St. Jude Children's Research Hospital, and Department of Microbiology, University of Tennessee Medical Units, Memphis, Tennessee 38101

Received for publication 21 September 1967

The growth and envelopment processes of three representative herpesviruses, equine abortion, pseudorabies, and herpes simplex, were examined in baby hamster kidney (BHK 21/13) cells by bioassay (plaque-forming units) and electron microscopy. The envelopment process was identical for all three viruses. After assembly in the nucleus, the nucleocapsid acquired an envelope by budding from the inner nuclear membrane. This membrane was reduplicated as the enveloped particle was released so that the budding process did not result in disruption of the continuity of the nuclear membrane. That portion of the nuclear membrane which comprised the viral envelope was appreciably thicker than the remainder of the membrane and exhibited numerous projections on its surface. Once enveloped, the viral particles were seen in vesicles and vacuoles in the cell cytoplasm. These appeared to open at the cytoplasmic membrane, releasing the virus from the cell. There was no detectable difference in the size or appearance of enveloped particles in intra- or extracellular locations.

The nucleus has been established as the site of herpesvirus assembly (9, 14, 19). Virus particles in the nucleus possess a single limiting membrane, the capsid; cytoplasmic and extracellular particles are surrounded by a second limiting membrane, the envelope (1, 9). Although some workers have reported that the envelope is derived from cytoplasmic elements (4, 17), most published reports have indicated that envelopment occurs at the nuclear membrane (1, 5, 8, 12, 15-17); we reported (2) that equine abortion virus (EAV) is enveloped at the inner nuclear membrane regardless of the cell line used to propagate the virus.

To clarify the sequence of events in the envelopment process, we investigated three representative herpesviruses, EAV, pseudorabies (Pr), and herpes simplex (H4), grown in a single cell line (BHK 21/13). The growth of these viruses was examined by bioassay and electron microscopy to determine the manner and sequence of envelopment and to ascertain whether the process was similar for all three viruses.

¹ Presented in part at the annual meetings of the American Society for Microbiology, New York, N. Y., May 1967, and the Tissue Culture Association, Philadelphia, Pa., June 1967.

² The results presented here comprise a portion of a dissertation submitted to the University of Tennessee Medical Units in partial fulfillment of requirements for the degree of Doctor of Philosophy.

The envelopment process was found to be the same for all three viruses. Envelopment occurred at the inner nuclear membrane, and virus particles did not acquire additional membranes as they passed through the cytoplasm and were released from the cell. There was a morphological alteration in that portion of the nuclear membrane which enveloped the virus.

MATERIALS AND METHODS

Viruses. The H4 strain of herpes simplex (6), the Pr strain of pseudorabies virus (7), and the Kentucky D strain of EAV (13) were used.

Cells and media. Monolayer cultures of baby hamster kidney cells, BHK 21/13 (18), were used for growth of all three viruses. The cells were routinely grown in Eagle's minimal essential medium, supplemented with 10% fetal calf serum. After virus infection, the same medium was used with 2% fetal calf serum.

Sequential studies. Confluent monolayers of BHK 21/13 cells in plastic petri dishes (60 by 15 mm) were infected with EAV and H4 viruses at an input multiplicity of 3 to 5 plaque-forming units (PFU) per cell, and with Pr virus at an input multiplicity of approximately 10 PFU per cell. After 1 hr of adsorption at 37 C, monolayers were washed with phosphate-buffered saline (PBS; 3), and 5 ml of maintenance medium was added. Duplicate cultures were taken at selected intervals after infection, one for electron microscopy and the other for assay of released (RV) and cell-associated (CAV) virus. Plaque assays were carried out as previously described (2),

FIG. 1. Representative growth curve of pseudorabies (Pr) virus in BHK 21/13 cells. RV indicates released virus (●); CAV indicates cell-associated virus (▲).

except that 1 ml of 1% agar and 4 ml of liquid maintenance medium were added to the H4 and Pr cultures. Plaques on these cultures were made visible by pouring off the soft agar and staining with a 2% aqueous solution of crystal violet.

Electron microscopy. Cells were scraped from the surface of infected and uninfected cultures and prepared for electron microscopy as described previously (2). For negative stains, virus was sedimented from the supernatant fluid of infected cultures and resuspended in one-tenth the original volume of PBS. One drop of this suspension was placed on a carbon-coated copper grid and allowed to stand for 1 min. The excess fluid was drained off and the grid was stained with 2% phosphotungstic acid (PTA; pH 7.2) for an additional 1 min. The grids were examined immediately in a Siemens Elmiskop I electron microscope.

RESULTS

Virus growth curves. Samples were taken simultaneously for bioassay (PFU) and electron microscopy in order that morphological events might be related to the growth stage of the virus.

A representative growth curve for Pr virus in BHK 21/13 cells is shown in Fig. 1. Growth curves for H4 and EAV viruses in these cells were similar, except that the increase in both CAV and RV occurred 1 to 3 hr later in the growth cycle. With all three viruses, the increase in both CAV and RV occurred 5 to 8 hr after infection; by 10 to 15 hr, more than 80% of the total virus was released from the cells.

Viral envelopment. The envelopment process was identical for all three viruses, so it will be discussed as one entity with representative micrographs to illustrate the various aspects.

Envelopment of virus at the inner nuclear membrane could be seen in sections taken coin-

FIG. 2. Stages in the budding process of herpesviruses from the inner nuclear membrane of BHK 21/13 cells. Note the thickening of the nuclear membrane as it surrounds the viral particle. Nucleus is at lower right in all micrographs. Bar indicates 0.1 μ . (a) H4 virus 21 hr after infection. $\times 90,000$. (b) EAV 12 hr after infection. $\times 110,000$. (c) Pr virus 12 hr after infection. $\times 144,000$.

FIG. 3. *BHK 21/13* cell 18 hr after infection with *H4* virus. Note the intranuclear vacuoles, most of which are continuous with the perinuclear cisterna. Viral particles in both intranuclear and intracytoplasmic vacuoles are enveloped. Bar indicates $0.5\ \mu$. $\times 22,400$.

cident with or shortly before the increase in RV (5 to 8 hr after infection). When viral capsids, with or without nucleoids, came into contact with the inner nuclear membrane, this membrane

became noticeably thicker at the point of contact (Fig. 2a, c; Fig. 4b, d). The particles became completely enclosed in this thickened membrane and appeared to pinch off, leaving the nuclear mem-

FIG. 4. Nuclear vacuoles in BHK 21/13 cells 12 hr after infection with H4 virus. Viral particles within the vacuoles are enveloped while those outside are unenveloped (a). Viral particles may be seen budding into the vacuoles (arrows, b and d). On occasion, envelopes containing multiple particles are seen (arrow, c). The vacuoles are often continuous with the perinuclear cisterna (d). The nucleus is in the lower portion of all micrographs. Bar indicates 0.5 μ . (a, d) $\times 33,000$. (b, c) $\times 44,000$.

brane intact in a manner analogous to the formation of pinocytotic vesicles at the plasma membrane. As the thickened membrane surrounded the virus particle, projections were seen on both the inner and outer aspects of the envelope (Fig. 8a). Once released, the enveloped particles were seen lying free in the perinuclear cisterna (Fig. 3).

Occasionally, vesicles containing enveloped nucleocapsids were present within the nuclei and cytoplasm of infected cells (Fig. 3). This was particularly true of cells infected with herpes simplex virus. Infected cells (Fig. 3) often exhibited extensive infoldings of the nuclear membrane, and it is possible that these vesicles represent such infoldings cut in cross section. Unen-

veloped nucleocapsids were seen surrounding these vesicles, whereas nucleocapsids inside were invariably enveloped (Fig. 4a). Nucleocapsids were often seen budding into a vesicle (arrows, Fig. 4b, d). Although most of the nucleocapsids within the vesicles were individually enveloped, envelopes surrounding multiple particles were occasionally seen (arrows, Fig. 4c). Vesicles, containing enveloped particles and continuous with the perinuclear cisterna, were also seen (Fig. 3, 4d).

The presence of budding particles was relatively rare in sections taken early in the growth cycle (8 to 10 hr). This was attributed to the small sample obtained by the ultrathin-sectioning tech-

FIG. 5. BHK 21/13 cells 19 hr after infection with Pr virus, showing alignment of particles along the inner nuclear membrane. Nucleus is at the bottom of both micrographs. Bar indicates $0.5\ \mu$. (a) $\times 44,000$. (b) $\times 36,000$.

FIG. 6. BHK 21/13 cells 18 hr after infection with H4 virus. Virus particles are apparently acquiring envelopes by budding from the cytoplasm into the perinuclear cisterna. Nucleus is at the bottom. Bar indicates $0.5\ \mu$. $\times 51,000$.

nique. However, at later time intervals (18 hr or later), the presence of such budding particles was much more prevalent, ranging from a particle situated every few hundred millimicrons (Fig. 5a) to alignments where particles were adjacent to

each other along large segments of the nuclear membrane (Fig. 5b).

Unenveloped particles were rarely seen in the cytoplasm; when this occurred, it was always late (24 hr or later after infection). Such particles

FIG. 7. Enveloped virus particles in the cytoplasm of BHK 21/13 cells are seen enclosed in small vacuoles containing one or two particles (arrow, a) or in larger vacuoles containing many enveloped particles (asterisk, a). These vacuoles are sometimes quite near the cytoplasmic membrane (arrow, b). There are no obvious morphological differences in enveloped particles in intra- or extracellular locations (b). Bar indicates $0.5\ \mu$. (a) H4 virus 12 hr postinfection. $\times 48,000$. (b) Pr virus 8 hr postinfection. $\times 96,000$.

FIG. 8. Extracellular Pr virus as demonstrated by thin sectioning (a) and negative staining (b). Note difference in appearance of envelope. Unit membrane structure is apparent in a (arrow). Projections on envelope are seen by negative staining (arrow, b). Bar indicates $0.1\ \mu$. (a) $\times 95,000$. (b) $\times 186,000$.

were sometimes seen in the process of acquiring envelopes at cytoplasmic membranes and were found, on occasion, to be budding from the cell cytoplasm through the outer lamella into the perinuclear cisterna (Fig. 6). It should be noted that, when such particles were seen, breaks in the nuclear membranes were also present.

After release from the nucleus, the enveloped particles were found in the cytoplasm in vacuoles, sometimes containing several particles (asterisk, Fig. 7a), or enclosed in an additional membrane which was apparently derived from cytoplasmic elements (arrow, Fig. 7a). Occasionally, enveloped particles were seen in vacuoles near the cytoplasmic membrane (arrow, Fig. 7b).

There was no difference in overall size of enveloped particles regardless of whether they were found in the perinuclear cisterna, the cytoplasm, or outside the cell. They ranged from 140 to 160 μ m in diameter in all locations. The morphology of extracellular enveloped particles (Fig. 7b) was identical to that of such particles inside the cell (arrow, Fig. 7b). The envelope consisted of a unit membrane which measured 10 to 15 μ m in thickness when it could be clearly discerned (arrow, Fig. 8a). The projections surrounding this membrane usually made it appear much thicker than this. In negatively stained preparations (PTA) of purified virus, the envelopes appeared much more irregular than in sections (Fig. 8b). This is possibly a result of the PTA treatment or of the drying effect, although samples of virus treated with either glutaraldehyde or osmic acid and suspended in a volatile buffer (0.1 M ammonium acetate) also exhibited this appearance. The projections noted on the viral envelopes in thin section were also seen on negatively stained envelopes (arrow, Fig. 8b).

DISCUSSION

The possibility that nuclear sites were involved in the envelopment of herpesviruses was initially raised by Falke et al. (5), who studied herpes simplex in HeLa cells, and by Morgan et al. (9), who examined this virus in the chorioallantoic membrane of chicken embryos and later in human amniotic and HeLa cell cultures (10).

Further evidence for the mode of envelopment was provided by the *in vivo* demonstration of nuclear budding in herpes simplex virus-infected mouse brain (S. Luse, P. Friedman, and M. Smith, *Am. J. Pathol.* 46:8A, 1965). Nuclear budding has also been reported for human cytomegalovirus in human lung fibroblast cells (1) and in human embryonic skin cells (8). Mouse (15) and guinea pig cytomegaloviruses (12) have also been shown to be enveloped at this site. It has been demonstrated that envelopment of EAV

occurs at the inner nuclear membrane regardless of the cell strain in which it is grown (2). From these reports and the present study, it appears clear that the primary site of envelopment of the herpesviruses is at the inner nuclear membrane.

The possibility exists that there are other sites of envelopment for the herpesviruses. Epstein (4) demonstrated budding of herpes simplex virus into cytoplasmic vacuoles and from the cytoplasmic membrane of cultured cells late in infection (48 to 84 hr), and Siminoff and Menefee (17) reported that envelopment occurred in the vicinity of the Golgi apparatus. Late in infection we have observed rare unenveloped particles in the cytoplasm of infected cells; when this occurred, breaks in the nuclear membrane could usually be detected. Occasionally, such particles appeared to be acquiring envelopes at sites other than the inner nuclear membrane (Fig. 6). No relationship of virus particles to the Golgi apparatus was seen. Shipkey et al. (16), who very clearly demonstrated the budding of herpes simplex virus from the inner nuclear membrane of HEP-2 cells, never observed enveloped particles in the cytoplasm, even as late as 7 days after infection. On the basis of our observations, it seems probable that the envelopment process is a random one and that herpesvirus particles become enveloped wherever they come into contact with a cell membrane. Unless the nucleus is disrupted, the nuclear membrane is the first such membrane encountered by the virus particle and therefore is the primary site of herpesvirus envelopment.

There was morphological alteration (Fig. 2a-c) of the nuclear membrane at the site of envelopment. This consisted of a marked thickening of the membrane and the appearance of projections on its surface. Once the nucleocapsid had acquired its envelope at the nuclear membrane, there was no alteration in size or appearance of particles in the cytoplasm or extracellularly. Enveloped particles in all locations were between 140 and 160 μ m in diameter, and there was no evidence of an additional membrane associated with extracellular virus particles. Some workers (16) have described "triple membraned" particles in the cytoplasm of herpesvirus-infected cells. We feel that this term is misleading. Only two of these, the capsid (which often appears as a membrane when cut in cross section) and the envelope, are part of the viral particle proper. If there is a third membrane, it is probably the wall of the vesicle or vacuole which sequesters the particle so that it does not lie free in the cytoplasm.

On the basis of the results presented here, the following mechanism for the envelopment and release of herpes viruses from infected cells is proposed. After assembly in the nucleoplasm, the

viral particles migrate to the nuclear membrane, where they are enclosed in an envelope derived from this membrane. This envelopment is the result of a budding process, and the nuclear membrane is left intact. The enveloped particles, either singly or in groups, are enclosed in vacuoles or vesicles derived either from the outer lamella of the perinuclear cisterna or from a lamella of the endoplasmic reticulum. These do not really represent different sites, as the cisternae of the endoplasmic reticulum are continuous with the perinuclear cisterna (11). The enclosed particles migrate to the plasma membrane, where the vacuole or vesicle opens and releases the enveloped viral particle(s), maintaining the continuity of the plasma membrane. This may represent a sort of reverse phagocytosis.

ACKNOWLEDGMENTS

The excellent technical assistance of Sara Clark is gratefully acknowledged.

This investigation was supported by Public Health Service research grant AI 05765 from the National Institute of Allergy and Infectious Diseases, and by American Lebanese Syrian Associated Charities (ALSAC).

LITERATURE CITED

1. BECKER, P., J. L. MELNICK, AND H. D. MAYOR. 1965. A morphologic comparison between the development stages of herpes zoster and human cytomegalovirus. *Exptl. Mol. Pathol.* **4**:11-23.
2. DARLINGTON, R. W., AND C. JAMES. 1966. Biological and morphological aspects of the growth of equine abortion virus. *J. Bacteriol.* **92**:250-257.
3. DULBECCO, R., AND M. VOGT. 1954. Plaque formation and isolation of pure lines with poliomyelitis viruses. *J. Exptl. Med.* **99**:167-182.
4. EPSTEIN, M. A. 1962. Observations on the mode of release of herpes virus from infected HeLa cells. *J. Cell Biol.* **12**:589-597.
5. FALKE, D., R. SIEGERT, AND W. VOGELL. 1959. Elektronen mikroskopische Befunde zur Frage der Doppelmembranbildung des Herpes-simplex-virus. *Arch. Ges. Virusforsch.* **9**:484-496.
6. KAPLAN, A. S. 1957. A study of herpes simplex virus-rabbit kidney cell system by the plaque technique. *Virology* **4**:435-457.
7. KAPLAN, A. S., AND A. E. VATTER. 1959. A comparison of herpes simplex and pseudorabies viruses. *Virology* **7**:394-407.
8. MCGAVRAN, M. H., AND M. G. SMITH. 1965. Ultrastructural, cytochemical and microchemical observations on cytomegalovirus (salivary gland virus) infection of human cells in tissue culture. *Exptl. Mol. Pathol.* **4**:1-10.
9. MORGAN, C., S. A. ELLISON, H. M. ROSE, AND D. H. MOORE. 1954. Structure and development of viruses as observed in the electron microscope. I. Herpes simplex virus. *J. Exptl. Med.* **100**:195-202.
10. MORGAN, C., H. M. ROSE, M. HOLDEN, AND E. P. JONES. 1959. Electron microscopic observations on the development of herpes simplex virus. *J. Exptl. Med.* **110**:643-656.
11. PALADE, G. E. 1955. Studies on the endoplasmic reticulum. II. Simple disposition *in situ*. *J. Biophys. Biochem. Cytol.* **1**:567-582.
12. PATRIZI, G., J. N. MIDDELKAMP, AND C. A. REED. 1967. Reduplication of nuclear membranes in tissue culture cells infected with guinea pig cytomegalovirus. *Am. J. Pathol.* **50**:779-790.
13. RANDALL, C. C., AND L. A. LAWSON. 1962. Adaptation of equine abortion virus to Earle's L cells in serum free medium with plaque formation. *Proc. Soc. Exptl. Biol. Med.* **110**:487-489.
14. REISSIG, M., AND J. L. MELNICK. 1955. The cellular changes produced in tissue culture by herpes B virus correlated with the concurrent multiplication of the virus. *J. Exptl. Med.* **101**:341-351.
15. RUEBNER, B. H., K. MIYAI, R. J. SLUSSER, P. WEDEMYER, AND D. N. MEDEARIS, JR. 1964. Mouse cytomegalovirus infection. An electron microscopic study of hepatic parenchymal cells. *Am. J. Pathol.* **44**:799-821.
16. SHIPKEY, F. H., R. A. ERLANDSEN, R. B. BAILEY, V. I. BABCOCK, AND C. M. SOUTHAM. 1967. Virus biographies. II. Growth of herpes simplex virus in tissue culture. *Exptl. Mol. Pathol.* **6**:39-67.
17. SIMINOFF, P., AND M. G. MENEFEE. 1966. Normal and 5-bromo-deoxyuridine-inhibited development of herpes simplex virus. *Exptl. Cell Res.* **44**:241-255.
18. STOKER, M. G. P., AND I. MACPHERSON. 1964. Syrian hamster fibroblast cell line BHK 21 and its derivatives. *Nature* **203**:1355-1357.
19. STOKER, M. G. P., K. M. SMITH, AND R. W. ROSS. 1958. Electron microscopic studies of HeLa cells infected with herpes virus. *J. Gen. Microbiol.* **19**:244-249.