

Biological and Morphological Aspects of the Growth of Equine Abortion Virus

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ABSTRACT

DARLINGTON, R. W. (St. Jude Children's Research Hospital, Memphis, Tenn.), AND C. JAMES. Biological and morphological aspects of the growth of equine abortion virus. *J. Bacteriol.* **92**:250-257. 1966.—The growth of equine abortion virus (EAV) was studied by bioassay and electron microscopy in L-cell monolayer and suspension cultures, and in HeLa and BHK 21/13 cell monolayers. Results of virus assay (plaque-forming units) indicated that production of cell-associated virus (CAV) began at 6 to 9 hr after infection in all of the cell strains used. Virus release occurred 1 to 2 hr later. By 15 to 20 hr after infection, the amount of released virus (RV) equaled or surpassed that of CAV in all cells other than the HeLa cells, where the amount of RV did not equal CAV until 48 hr after infection. Electron microscopy of infected cells revealed no differences in the morphology of virus development in any of the cells used. Developing virus particles were first detected in cell nuclei at 9 hr after infection. At 12 hr, virus particles could be seen budding from the inner nuclear envelope. Budding into cytoplasmic vacuoles was not seen. Budding virus, virus in cytoplasmic vacuoles, and extracellular virus were all approximately 145 μ in diameter, and were indistinguishable morphologically. These results indicated that EAV is quite similar to herpes simplex virus with respect to growth and morphology, and that the inner nuclear membrane is the principal site of virus envelopment.

Reports of the morphological development of herpes simplex virus have been reconstructions based primarily on sections taken late in the infection (8, 16). The present study was undertaken to determine the sequence of events, particularly during the early part of the logarithmic-growth phase, of a similar herpesvirus. This was equine abortion virus (EAV), which has also been called equine herpesvirus I (19) or equine rhinopneumonitis virus (4).

The sequential development of EAV was examined by use of bioassay and electron microscopy in L-cell monolayer and suspension cultures. HeLa and baby hamster kidney (BHK 21/13) cells were also used to ascertain whether the host cell played a significant role in the pattern of virus development. The results of these investigations provide information about EAV synthesis and envelopment, and establish it as a group A herpesvirus (14).

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MATERIALS AND METHODS

The L cell-adapted Kentucky D strain of EAV (20) was used. Virus in plaque suspension isolated from an L-cell monolayer was used as seed to prepare stock virus.

Cells. Three continuous cell lines were used. LM mouse fibroblast cells were grown in suspension in yeast extract-lactalbumin hydrolysate medium (YELP) with added Methocel (13), and as monolayers in 90% YELP and 10% calf serum. Monolayer cultures of HeLa (11) and BHK 21/13 (25) cells were grown in 90% minimal Eagle's medium (MEM; 7) containing Hanks salt solution and 10% calf serum.

Plaque assay. All plaque assays were done on L-cell monolayers. Confluent monolayers of cells grown in 90% YELP and 10% calf serum were washed with phosphate-buffered saline (PBS; 5) and inoculated with 0.2 ml of virus. After an adsorption period of 1 hr at 37 C the inoculum was removed, and the cultures were washed three times with PBS and overlaid with 5 ml of medium. The overlay medium consisted of 98% YELP, 2% calf serum, and 1.1% (w/v) agar. The cultures were incubated at 37 C in an atmosphere of 5% CO₂ in air. After 3 days, a second overlay containing 0.06% (w/v) neutral red in addition to the

other ingredients was added, and plaques were counted 2 days later.

Electron microscopy. Cell pellets obtained from either monolayer or cell suspension cultures were fixed for 10 min in pH 7.2 phosphate buffer containing 5% glutaraldehyde. They were then washed with phosphate buffer, cut into 1-mm square pieces, and fixed for an additional 20 min in 1% osmic acid in Milonig's (15) phosphate buffer. The blocks were rapidly dehydrated in graded alcohols and embedded in modified Maraglas (9). Sections were cut with glass knives on an LKB microtome and collected on Formvar-coated copper grids. Sections were stained with uranyl acetate (24) and lead citrate (22), and were examined in a Siemens Elmiskop I electron microscope.

Sequential studies. Monolayer cultures of L, BHK 21/13, and HeLa cells containing approximately 2×10^6 cells per culture were infected at input multiplicities of 5 plaque-forming units (PFU) per cell, and incubated at 37 C for 1 hr to allow for virus attachment. The cultures were then washed, and 5 ml of maintenance medium was added to each plate. MEM with 2% calf serum was used for HeLa and BHK 21/13 cells, and YELP with 2% calf serum was used for L cells. Both released virus (RV) and cell-associated virus (CAV) plaque assays were made on duplicate cultures of each cell type harvested at various time intervals after infection. Fluid was harvested and centrifuged at 1,500 rev/min for 5 min. The supernatant fluid, stored at -60 C, was assayed for RV. The cell layers were washed three times with PBS, suspended in 5 ml of maintenance medium, and stored in sealed glass ampoules at -60 C. The cells were subsequently subjected to two cycles of freezing and thawing to release CAV. The fluid was also subjected to two cycles of freezing and thawing for consistency of treatment of preparations. For growth studies in suspended L-cell cultures, cells were collected by centrifugation and resuspended in virus at a ratio of 5 PFU per cell. Adsorption at 37 C for 1 hr was followed by three washings and resuspension of cells in 100 ml of growth medium (YELP plus Methocel), resulting in a final concentration of 2×10^6 cells per milliliter. Duplicate 5-ml samples were taken at intervals of time after infection comparable to those taken for monolayer cultures. The fluid and cells were treated in essentially the same manner as monolayer cultures for obtaining released and cell-associated virus. Samples were taken for electron microscopy at the same time intervals as samples for virus assay.

RESULTS

Virus growth curves. Representative EAV growth curves in L-cell monolayer and suspension cultures are shown in Fig. 1A and B, respectively. In monolayer cultures (Fig. 1A), the initial increase in both CAV and RV occurred between 6 and 9 hr after infection. The titer of RV equaled that of CAV at about 15 hr, and both titers continued to rise throughout the course of the experiment (48 hr). In suspension cultures (Fig. 1B), the initial rise of CAV also occurred between

6 and 9 hr after infection. However, the rise in titer of RV was not seen until 9 to 12 hr after infection. As in monolayer cultures, RV equaled CAV at 15 hr, and continued to rise until about 36 hr. CAV reached its peak titer by 12 hr and did not increase thereafter. The growth curve of EAV in suspension cultures of L cells (Fig. 1B) demonstrates the usefulness of such a system. It was possible to obtain very low background levels of input virus, so that the sequence of virus synthesis and release was more clearly delineated. At 6 hr after infection, there was no demonstrable virus in either cells or supernatant fluid. By 9 hr, there was a 10^4 PFU/ml increase in CAV with no detectable virus in the supernatant fluid. At 12 hr, virus (10^4 PFU/ml) was present in the supernatant fluid.

EAV growth in BHK 21/13 (Fig. 1C) and HeLa (Fig. 1D) cells did not differ markedly from growth in L cells with regard to appearance of detectable CAV and RV. Late in the infection of BHK 21/13 (Fig. 1C) cells, a greater proportion of the virus was released. Conversely, in HeLa cells (Fig. 1D), most of the virus remained cell-associated until quite late in the infection (48 hr).

Morphology of virus development. As there were no differences at the ultrastructural level in the pattern of infection in any of the cell lines, representative micrographs from all cells will be used to illustrate various aspects of virus development.

No morphological evidence of virus infection was seen at 6 hr, but, at 9 hr, developing virus could be seen within the cell nuclei (Fig. 2). The predominant form consisted of two concentric rings. At later time intervals, the inner ring apparently condenses into the nucleoid, resulting in a particle with a nucleoid and a single membrane. Another form seen in the nucleus had a single limiting membrane with a cross-shaped electron-lucent area in the center (Fig. 3). The inner portion of these particles may also condense into nucleoids (Fig. 4). Virus synthesis did not appear to be confined to any specific area of the nucleus. Developing particles were dispersed throughout and had no predilection for sites near the marginated chromatin. The developing particles measured from 85 to 90 m μ in diameter.

At 12 hr after infection, virus could be seen budding from the inner nuclear membrane (Fig. 5). The envelope formed around the particles was clearly a continuation of the membrane which pinched off and thus maintained the integrity of the nuclear envelope. This budding process was the only method of egress from the

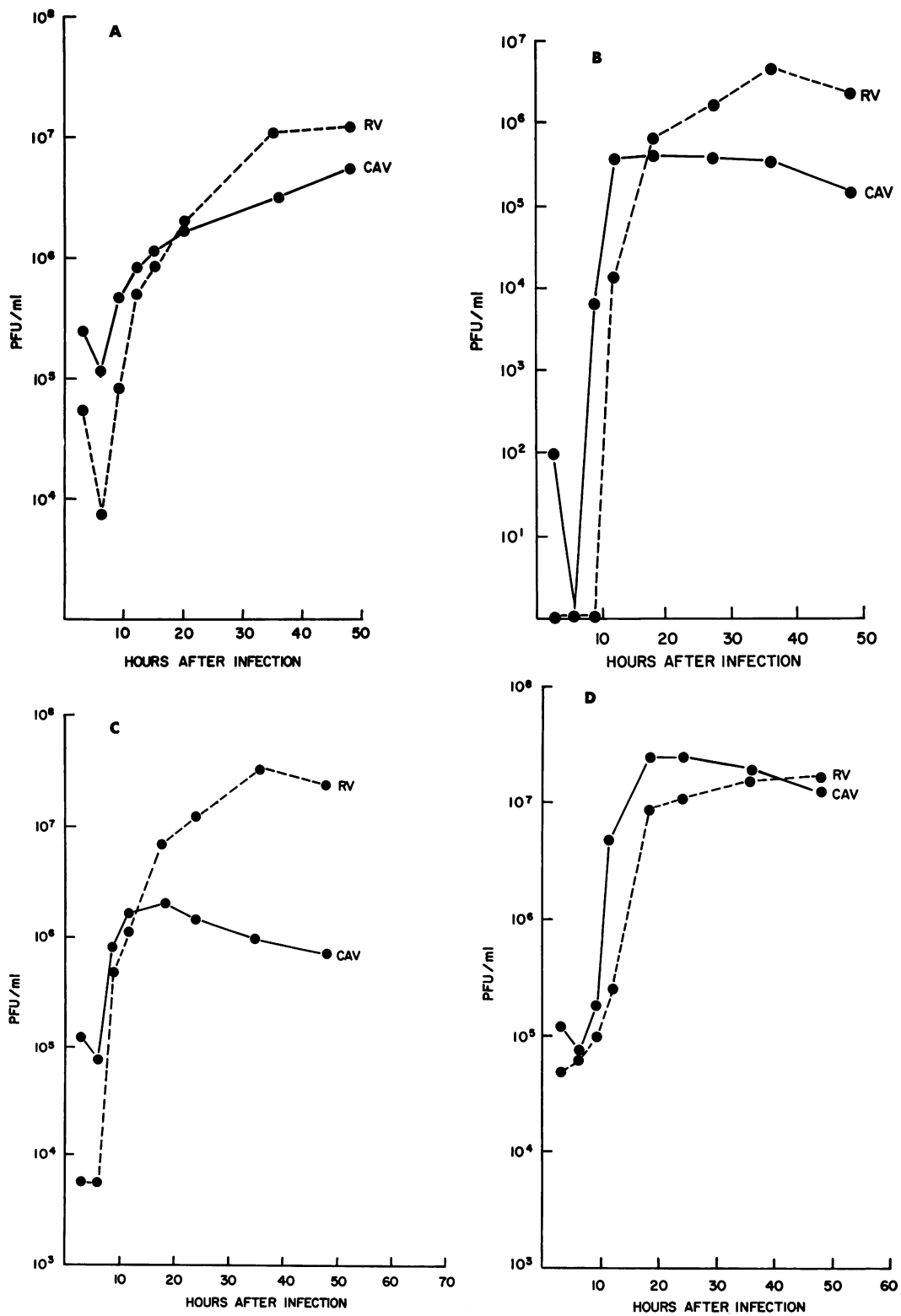


FIG. 1. EAV growth curves. (A) L-cell monolayer. (B) L-cell suspension. (C) BHK 21/13 cell monolayer. (D) HeLa cell monolayer. Solid line, cell-associated virus (CAV); broken line, released virus (RV). Assays of virus samples from all cells were done in L-cell monolayers.

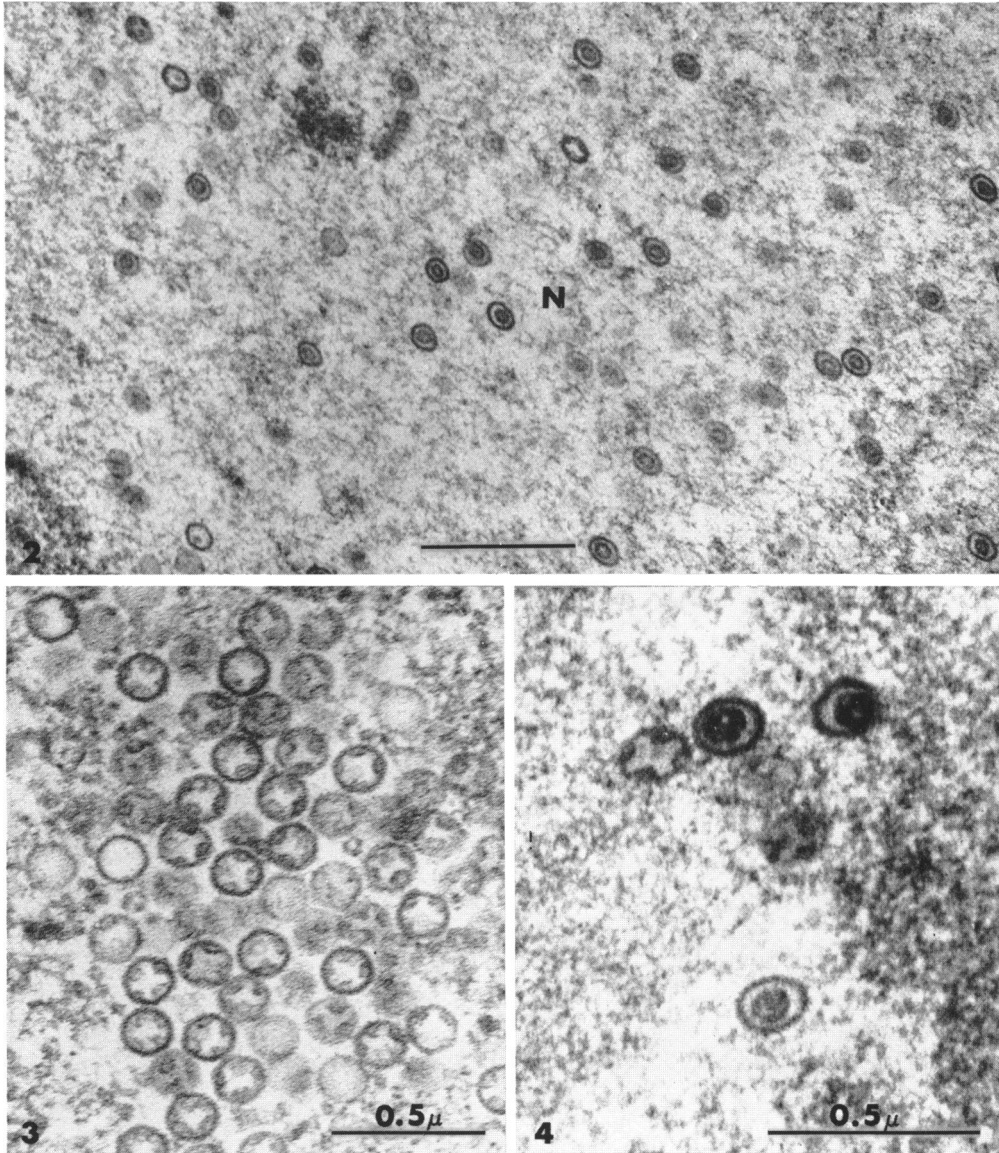


FIG. 2. Suspension of L cells 9 hr after infection. Portion of an infected nucleus showing developing virus particles. Majority of particles are the double-ringed form. $\times 20,000$. (Bar equals 1μ in all micrographs unless otherwise indicated. N = nucleus.)

FIG. 3. Suspension of L cells 24 hr after infection. Portion of an infected nucleus containing cross-shaped structures. $\times 40,000$.

FIG. 4. Suspension of L cells 10 hr after infection. Area of nucleus with cross-shaped and double-ringed particles. The two upper right-hand particles contain partially condensed nucleoids. $\times 55,000$.

nucleus observed. At this time virus could also be seen in vacuoles which appeared to communicate with the perinuclear cisterna (Fig. 6 and 7, arrows), and extracellularly. Fine fibrillar projections could be seen on the outer surface of the particle both as it budded from the nuclear

envelope (Fig. 5), and as it was observed in the cytoplasm (Fig. 6), and outside the cell (Fig. 8). Virus particles from all of these locations were 135 to 145 $m\mu$ in diameter. There appeared to be no increment in size once the virus was released from the nuclear membrane. The nuclear budding

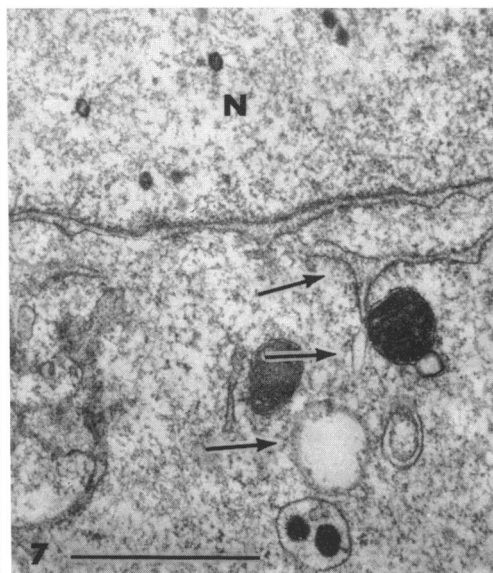
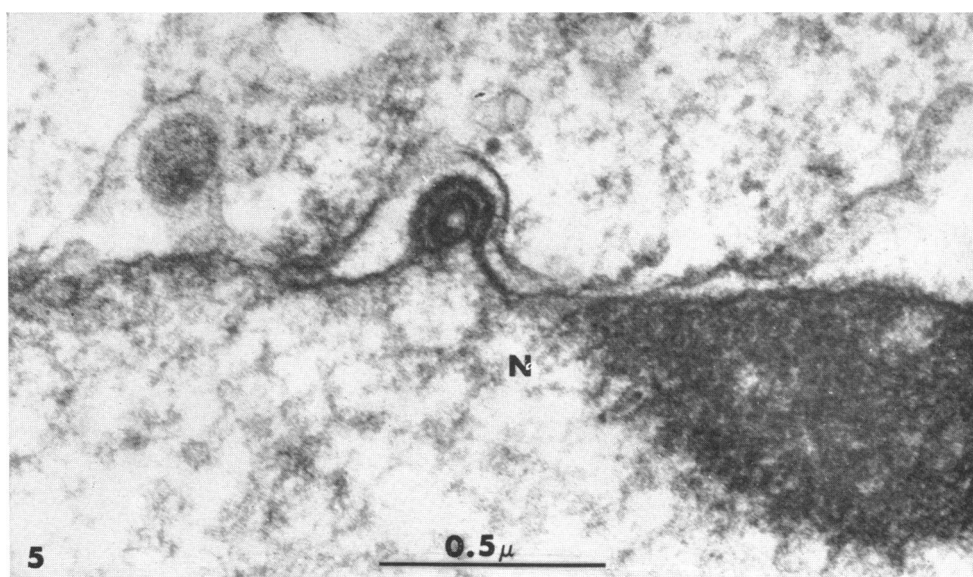


FIG. 5. Monolayer HeLa cell 12 hr after infection. Virus particle acquiring an envelope by budding from the nuclear membrane. Note fine fibrils on surface of envelope surrounding particle. $\times 60,000$.

FIG. 6. BHK 21/13 monolayer cell 12 hr after infection. Virus particles in cytoplasmic vacuole. A cisterna of the endoplasmic reticulum (arrow) appears to connect the perinuclear cisterna with the vacuole. $\times 30,000$.

FIG. 7. Monolayer L cell 18 hr after infection. Virus particles in cytoplasmic vacuole. Possible interconnecting cisternae of endoplasmic reticulum are marked by arrows. $\times 25,000$.

process and the appearance of extracellular virus occurred simultaneously and coincided with the increase in titer of extracellular virus.

The precise mechanism by which the virus left the cell could not be determined. Occasionally virus could be seen in indentations in the cyto-

plasmic membrane (Fig. 8, arrow), suggesting release. However, this could represent attachment and penetration rather than release.

Later in the infection (24 hr or more), many virus particles could be seen in the cytoplasm, both inside and outside of vacuoles. No evidence

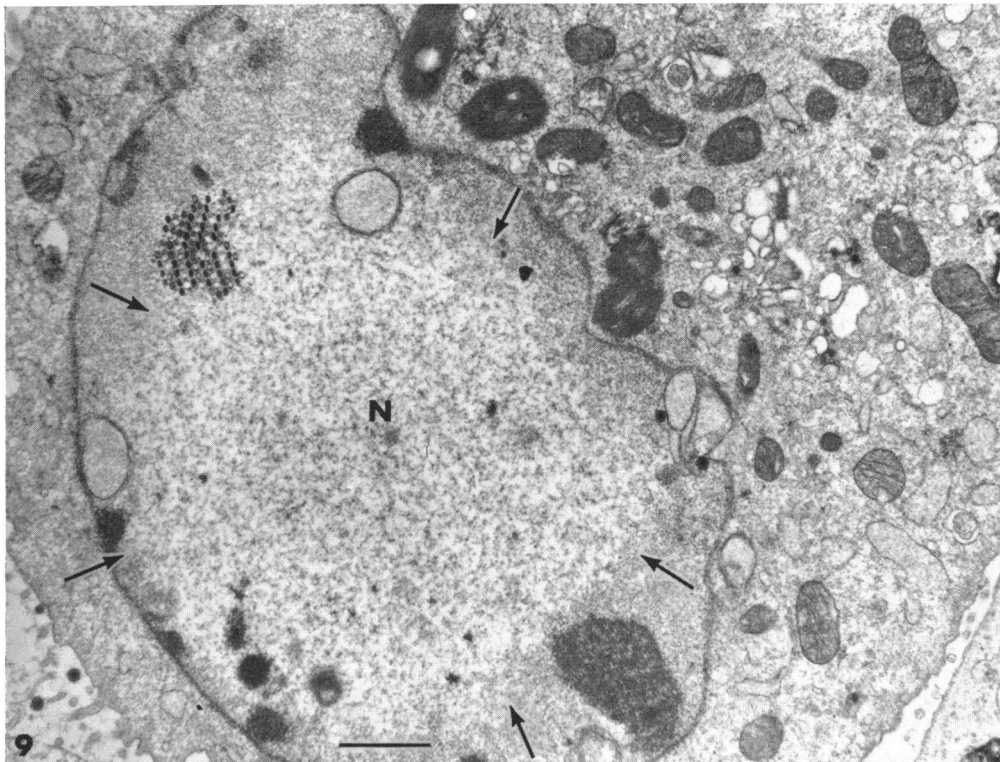
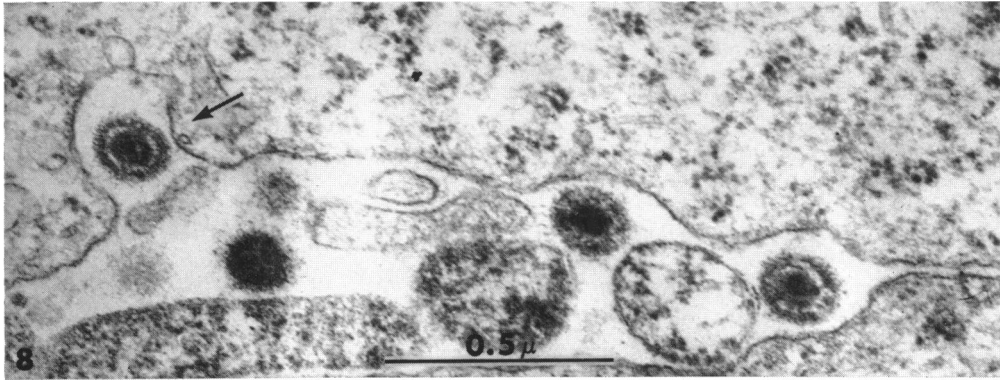


FIG. 8. BHK 21/13 monolayer cells 12 hr after infection. Extracellular virus. One particle (arrow) lies in an indentation of the cytoplasmic membrane. Note fibrillar projections on surface of virus particles. $\times 50,000$.

FIG. 9. Monolayer L cells 48 hr after infection. Inclusion area (arrows) is clearly delineated. There is a small crystal in the upper left-hand corner of the nucleus. The chromatin is fragmented and displaced to the periphery of the nucleus. The cell cytoplasm shows areas of vacuolation. $\times 11,500$.

of budding into cytoplasmic vacuoles could be found.

At 24 to 48 hr after infection, there was an occasional appearance of small crystals of virus within the nucleus (Fig. 9). The inclusion area (arrows) was also well delineated at these later times (Fig. 9). This consisted of an irregular area, somewhat less dense than the remainder of

the nucleoplasm. The inclusion was made up of scattered developing virus and irregular coarse filaments.

DISCUSSION

The evidence for inclusion of EAV as a herpesvirus is now quite firm. It contains deoxyribonucleic acid with 56% guanine plus cytosine

(3, 23), and has the characteristic enveloped nucleocapsid structure as shown by Plummer and Waterson (19). It also has an antigenic relationship, by complement-fixation test, to herpes simplex (18). Because of its wide host range and lack of infectivity for humans, it provides an excellent model system for investigation of this group of viruses.

Examination of the growth curves reveals that EAV falls into subgroup A (14) of the herpesviruses, of which herpes simplex is an example. The viruses of this group are readily released from the cell in contrast with group B herpesviruses (14), which remain largely cell-associated.

No attempt was made to examine the attachment and penetration stages of EAV-cell interaction in this study. Previous observations (Darlington, *unpublished observations*) have shown that this process is in no way different from that seen in herpes simplex virus infections (12).

This present study has shown that the morphological development as observed by electron microscopy correlates well with the development of PFU. The initial appearance of virus in the nucleus occurred at the same time as the rise in cell-associated infectivity, and the detection of extracellular virus was concurrent with the rise in PFU titer of released virus.

Virus synthesis occurs diffusely throughout the nucleus, and these observations suggest that the initial particle is a double-ringed form of which the inner ring condenses to form the nucleoid of mature particles. The cross-shaped form is probably a variant which also matures by condensation of the inner component into the nucleoid. This latter form has been reported in EAV-infected hamster liver (1) and in swine kidney cells infected with EAV (61). Virus particles appear to migrate to the nuclear membrane after they are synthesized.

The most interesting observation was the envelopment of virus at the nuclear envelope. This has been reported for herpes zoster and cytomegalovirus in tissue culture (2), for herpes simplex in infected mouse brain (S. Luse, P. Friedman, and M. Smith, *Am. J. Pathol.* **46**:8A, 1965), and for inclusion body rhinitis of swine (6). Morgan (16) suggested this mode of envelopment for herpes simplex, but Epstein (8) felt that envelopment occurred on entry into cytoplasmic vacuoles. However, these results were obtained from a reconstructed sequence of events from cells taken 48 hr or more after infection. Our experience was that sections of cells taken 24 hr or later after infection present a confused picture of the stage of virus development. Cell damage is extensive, and it is very

difficult to determine whether cytoplasmic virus is entering or leaving the cell.

These difficulties were eliminated by the sequential study which demonstrated several facts regarding the envelopment process. (i) The envelopment and release of virus occurs within the same time period; (ii) there is no size increment in virus from the budding process at the nuclear membrane to extracellular virus; and (iii) in many sections there is continuity between the perinuclear cisternae and cytoplasmic vacuoles. These findings show that budding from the inner nuclear envelope is the principal method for envelopment and that once this has occurred the virus is, in effect, outside the cell. Continuity of the perinuclear cisterna with the cisternae of the endoplasmic reticulum and of these cisternae with the cytoplasmic membrane has been reported (17). The available evidence suggests that once outside the nucleus the virus does not traverse another membrane system. As has been pointed out by others (10) and as we have observed, the continuity of the endoplasmic reticulum with the cytoplasmic membrane is rare, if indeed it does exist. These findings do not preclude other methods of virus envelopment but suggest that envelopment at the nuclear membrane is the principal one.

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