Morphology and Entry of Enveloped and Deenveloped Equine Abortion (Herpes) Virus

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Selective removal of the envelope of equine abortion (herpes) virus was accomplished by utilizing the nonionic detergent Nonidet P-40 followed by sonic treatment. The deenveloped particles differ significantly in size and buoyant density from the enveloped form. The cellular entry of purified enveloped and purified deenveloped virus was examined by electron microscopy during critical time periods. Both forms appeared to enter cells by a viropexis mechanism in which particles were engulfed by pseudopodia which either surround the virus and fuse with the cell membrane or to other pseudopodia, forming fusion vacuoles containing from one to numerous viral particles. This mode of entry was noted extensively at 5 min postinoculation. Deenveloped particles were apparently infectious only for hamsters, with a large inoculum being required. Contamination by enveloped forms was not noted after exhaustive search by electron microscopy.

Equine abortion virus (EAV) is a member of the herpes virus group, all of which consist of a deoxyribonucleic acid (DNA) core surrounded by a protein capsid enclosed by a lipoprotein envelope (7, 12, 17, 22, 29). The capsid is an icosahedron composed of 162 capsomeres arranged in a 5:3:2 axis of symmetry (18, 22, 34). The primary site of envelopment of herpes virus particles appears to be at the nuclear membrane, especially at the inner lamella, though the process may occur within the nuclear matrix, cytoplasm, or plasma membrane sites (4-6, 20).

Studies of the infectivity of normal enveloped virus and unenveloped particles have yielded conflicting results. Watson et al. (33) reported that both enveloped and unenveloped forms of herpes simplex virus were infectious for BHK-21 cells. Their results were based on plaque assay and particle-counting procedures which indicated that the number of infectious units exceeded the number of enveloped particles. They suggested that the excess infectious units were the result of unenveloped particles. Smith (27) subjected herpes simplex virus to CsCl density gradient centrifugation and found infectivity associated mainly with a band containing only enveloped forms. However, he assumed that CsCl either had no effect or did not selectively degrade one form of the virus in preference to the other, an assumption that was later shown to be invalid

(30-32) since CsCl was found to be more deleterious for naked particles than for enveloped forms. Spring and Roizman (31), utilizing enveloped particles of herpes simplex virus from the cytoplasm and unenveloped particles from the nuclei of infected cells, found that the envelope protects the nucleocapsid and accelerates adsorption to cells, "but is not inherently essential for infectivity." Further, Roizman et al. (26) reported that though nucleocapsids derived from the nuclear fraction of infected cells are infectious, they may be so due to some associated lipid-containing material which is either a small portion of the envelope or, more likely, is different from the outer envelope.

Conflicting results have also been reported concerning the mechanism of entry of herpes viruses into cells. Dales and Silverberg (3), suggested a viropexis mechanism whereby viruses gain entry into cells by phagocytosis. Concurrently, Morgan et al. (19) suggested an alternative mechanism whereby entry of the particle consists of attachment to the cell, digestion of the viral envelope at the cell surface, and passage of the capsid directly into the cytoplasm.

Application of a modification of Easterbrook's (9) procedure for the controlled degradation of vaccinia virus has enabled us to obtain purified deenveloped EAV particles. The studies reported here were primarily designed to examine the

mechanism of entry of enveloped and deenveloped particles.

MATERIALS AND METHODS

Virus and experimental animals. The strain of equine abortion virus used in this study and the method of its propagation in the Syrian hamster and in L-M cells have been described (1, 7, 21, 23, 24). Because large quantities of virus were necessary, the serum from the viremic stage of infected hamsters was employed rather than virus prepared from tissue culture supernatant fluids, since a much greater quantity of easily purified virus could be obtained. The use of this strain of virus appeared justified, as preliminary experiments on the method of entry with L-M cells or hamster-propagated virus revealed no apparent differences.

Virus purification. Routinely, 100 hamsters which had been inoculated intraperitoneally were anesthetized with ether 12 hr postinfection and exsanguinated, and the serum was separated from the clotted blood by centrifugation. The clot was washed three times with phosphate-buffered saline [PBS (8)], and each supernatant fluid wash was added to the serum. This mixture was passed through a graded series of membrane filters (Millipore Corp., Bedford, Mass.) of 1.2, 0.45, and 0.20 μ m porosity. After filtration the virus was pelleted by centrifugation at $40,692 \times g$ for 30 min by using the SW-25 rotor in a Spinco model L ultracentrifuge. The pellet was resuspended in a small volume of PBS and washed three times as above by centrifugation at $40,692 \times g$. Further purification was obtained by suspending the pellet in 0.5 ml of PBS and by centrifugation in either sucrose (30 to 52%, w/v) or potassium tartrate (specific gravity 1.16 to 1.28) density gradients. Gradients were centrifuged at $32,644 \times g$ for 2 hr in the Spinco model L ultracentrifuge with the SW-50 rotor. Fractions were collected and the densities were determined as described by Darlington and Randall (7). Purity of the preparations was monitored by electron microscopy.

Preparation and purification of deenveloped particles. Deenveloped particles were prepared from purified hamster-adapted EAV by a modification of the technique described by Easterbrook for controlled degradation of vaccinia virus (9). Preliminary experiments revealed that equal volumes of purified virus in PBS and 5% Nonidet P-40 (Shell Chemical Co., San Francisco, Calif.), incubated for 1.5 hr at 37 C, and then briefly sonically treated for 15 to 30 sec in a Reac Ultrasonic Disintegrator (Ultrasonic Industries, Inc., New York, N. Y.) gave optimal removal of envelope material from the virus. After the Nonidet treatment, the suspension was centrifuged at 20,000 rev/min in a Spinco model L ultracentrifuge for 30 min with the SW-50 rotor. The pellet was resuspended and washed three times in PBS. Final purification and determination of hydrated density was obtained as described in virus purification methods.

Radioactively labeled virus. To obtain ^3H -thymidine (^3H -TdR)-labeled virus, hamsters were injected intraperitoneally at 4 and 6 hr postinfection with 50

μC of ^3H -TdR (specific activity 14 c/mm) per injection. Virus was harvested and purified as described above. Stability of ^3H -TdR-labeled enveloped and deenveloped particles to deoxyribonuclease was determined essentially by the procedure described by Joklik (16).

Electron microscopy. Enveloped virus and deenveloped particles were prepared by the pseudoreplica technique of Sharp, as described by Hyde et al. (15), and stained with 0.5% uranyl acetate (UA) and 1.0% phosphotungstic acid (PTA). The progress of uncoating was followed at 15-min intervals during the Nonidet treatment by the pseudoreplica technique. Particle counts of virus were done by the sedimentation method of Smith and Benyesh-Melnick (28).

Because synchrony of adsorption could be more easily monitored in an in vitro system, L-M cell monolayers were employed for electron microscopic time-sequence studies on the entry of enveloped and deenveloped EAV into cells. Approximately 1.3×10^{10} to 1.5×10^{10} particles per ml of density gradient-purified virus was allowed to attach to previously chilled L-M cell monolayers in Leighton tubes (21) at 4 C for 30 min. The infected monolayers were brought rapidly to 37 C, incubated for 0, 5, 10, 15, 30, and 60 min and 24 hr, and fixed in situ without washing with 6.25% glutaraldehyde and 1% osmium tetroxide; they were then scraped and embedded in DOW-332-732 resin. Thin sections cut with a diamond knife were stained with uranyl acetate and lead citrate and examined with an RCA EMU3-G electron microscope.

Infectivity studies. Infectivity studies of virus from whole blood, density gradient-purified enveloped and

FIG. 1. Pseudoreplica of enveloped virus stained with PTA, showing icosahedral capsid enclosed by envelope possessing spikelike projections (arrow). $\times 200,000$.

FIG. 2. Pseudoreplica of EAV particles in process of losing envelope material after incubation for 45 min with Nonidet followed by sonic treatment. Some envelope material is free (Fig. 2a, arrow), whereas in others it is streaming away from the capsid. Figure 2b shows free envelope material (arrow) and a capsid which has lost its structural integrity. PTA stained. $\times 140,000$.

deenveloped particles were carried out in the Syrian hamster as described by Randall and Bracken (23) and Arhelger et al. (1). Prior to infection, pseudoreplica preparations were examined by electron microscopy to determine purity, and no evidence of envelope material was observed in the deenveloped samples. The virus was injected both intraperitoneally and intrahepatically. Infectivity was judged by three criteria: (i) death of the animals, (ii) histological examination of infected liver for the presence of type A intranuclear inclusion bodies, and (iii) electron microscopic examination of liver for the presence of virus. The LD_{50} determinations were calculated by the method of Reed and Muench (25).

RESULTS

Electron microscopy of deenveloped virus. For purposes of comparison, electron micrographs of enveloped virus are included. Figure 1 is typical of normal EAV. The envelope surrounding the capsid contains numerous projections (Fig. 1, arrow) which are similar to those reported in herpes simplex (3) and pseudorabies virus (5).

Figure 2 illustrates EAV particles from which the envelope has been loosened after 45 min of Nonidet and sonic treatment. Several particles are observed with the envelope streaming away from but still in apparent contact with the capsid (Fig. 2a). Envelope material is also present free of the capsid (Fig. 2a, 2b, arrows) and exhibits the characteristic projections present in the envelope of the normal virus (Fig. 1, arrow). A capsid which has lost its structural integrity is seen in Fig. 2b. The capsid is disarranged and appears broken. Such broken or structurally disarranged capsids are not uncommon both prior to and after gradient centrifugation of deenveloped material.

After Nonidet treatment for 90 min and purification by density gradient centrifugation, the deenveloped particles appear as shown in Fig. 3. They are hexagonal and are 90 to 100 nm in diameter, in contrast to the normal particle size of 160 to 170 nm. No evidence of envelope material is present, and the structural integrity of the particles appears to be essentially intact.

FIG. 3. Pseudoreplica showing deenveloped particles after 90 min of Nonidet treatment followed by sonic treatment and density gradient centrifugation. Arrow A indicates presence of fragmented capsid material. Arrow B shows various shaped bodies appearing superimposed on particles, with possible underlying background capsomeres. Micrograph is also representative of deenveloped particles used for inoculum in infectivity studies. Uranyl acetate stained. $\times 115,000$.

The remnants of several fragmented capsids are also present (Fig. 3, arrows a). The bodies of various shapes, which appear to be superimposed on the viral particles (Fig. 3, arrows b), may be the result of the detergent treatment causing dislocations of masses of capsomeres. The apparent underlying background of faintly staining capsomeres may be the result of partial peeling of the capsid, revealing subsurface structure, or may be an artifact of UA penetration.

Comparison of thin sections of enveloped virus (Fig. 4) and deenveloped virus (Fig. 5) illustrate that the typical unit membrane of the envelope surrounding the capsid of normal EAV (Fig. 4, arrow) is absent in the deenveloped form (Fig. 5). The nucleoids of both forms appear to be bar shaped (Fig. 4b and 5a, arrows), or curled or oval, and are consistent with other reports (3, 18–20). In Fig. 4a, there are two distinct capsids containing bar-shaped nucleoids

enclosed in a single envelope. Also, an additional bar-shaped nucleoid is evidenced but does not appear to be enclosed by an intact capsid.

Disruption of the structural integrity of de-enveloped virus is illustrated in Fig. 6b-6d. Capsomere breakage from the capsid (Fig. 6b) becomes evident at 45 min during the Nonidet treatment and results in distortion and loss of capsomeres (Fig. 6c, 6d). Comparison of intact (Fig. 6a) and broken particles (Fig. 6c, 6d)

FIG. 4. Thin sections of enveloped EAV stained with uranyl acetate and lead citrate, showing unit membrane of envelope (Fig. 4a, arrow) and bar-shaped nucleoid (Fig. 4b, arrow). $\times 160,000$.

indicate that when the particle breaks up it becomes flattened or spread and appears larger than intact virions (Fig. 6d, arrow).

Density of particles. Purified ^3H -thymidine-labeled enveloped and deenveloped virus were mixed together and subjected to centrifugation in sucrose and potassium tartrate density gradients. Separation of the envelope from the de-enveloped particles was easily achieved by this method and resulted in two distinct bands with densities of 1.18 and 1.22, respectively (Fig. 7). Electron microscopy showed that the upper band consisted of enveloped virus and the lower band of deenveloped particles. It was noted that sedimentation in potassium tartrate density gradients often caused clumping of the particles. Also, for infectivity studies, potassium tartrate was found to be toxic when injected into hamsters and it was necessary to wash particles three times to eliminate this effect.

Stability of virions to deoxyribonuclease. Electron microscopy of deenveloped virus revealed that a number of particles were broken or exhibited distorted capsomeres (Fig. 2b, 6b-6d), indicating that the detergent may have affected the stability of the virions. To examine this, the criteria of deoxyribonuclease sensitivity was utilized. Samples (1 ml) containing approximately 2.90×10^{11} viral particles, labeled with ^3H -TdR, were incubated at 37 C for 60 min in 0.01 M MgCl_2 and 0.01 M phosphate buffer (pH 7.0) containing 100 μg of deoxyribonuclease. After the treatment, 3% of the radioactivity of enveloped virions was rendered acid soluble, in contrast to 24% for purified deenveloped particles. A plausible explanation for this increase is that the Nonidet treatment, which causes breaks in the particles (Fig. 2b, 6b-6d), renders the viral DNA accessible to deoxyribonuclease.

Assay of infectivity of deenveloped virus. In this laboratory, EAV has been cultivated in the Syrian hamster for the past 12 years through more than 500 passages and is highly infectious (2, 7, 12). The hamster strain of EAV has been adapted to L-M cells in a somewhat involved manner (21, 24). Unfortunately, primary inoculation of L-M cells with hamster EAV results in an abortive infection without evidence of adaptation (Randall, unpublished data). Upon primary passage, electron microscopy revealed margination of chromatin and incomplete viral particles in the nucleus after incubation for 24 hr. Cytoplasmic viral particles were rarely evident. Subsequent serial passage revealed no evidence of adaptation. In contrast, deenveloped hamster EAV were seen to penetrate the cytoplasm, but no particles were present in the nucleus and no

FIG. 5. Thin sections of deenveloped EAV after 90 min of Nonidet treatment and 30 sec of sonic treatment; stained with uranyl acetate and lead citrate. Capsids are devoid of envelope and possess different forms of nucleoid material. Arrow in Fig. 5a indicates bar-shaped nucleoid. $\times 160,000$.

FIG. 6. Phosphotungstic acid stain of deenveloped EAV taken after 45 min of Nonidet treatment showing intact and broken capsids. The capsid of the particle in Fig. 6a is intact, whereas capsomere breakage appears slight in Fig. 6b and more extensive in Fig. 6c. Figures 6c and 6d show broken particles which appear flattened and larger than the intact capsid (Fig. 6d, arrow). $\times 200,000$.

chromatin changes were observed. The infectivity of deenveloped hamster virus for the hamster was then assayed.

Hamsters were inoculated intraperitoneally with 1.0-ml portions containing a known number of viral particles from whole blood, purified enveloped virus and purified deenveloped virus. The LD₅₀ per milliliter and the number of viral particles per LD₅₀ were determined (7) for each preparation. Table 1 shows a striking loss of infectivity of enveloped and deenveloped preparations when compared with the titer of whole blood. Concurrent with the reduction of LD₅₀ titer per milliliter, there is a corresponding increase in the number of viral particles per LD₅₀, amounting to an approximate 4-log increase in the number of particles per LD₅₀ between each of the two preparations, respectively.

Histological examination of livers biopsied within 30 min after death of the animals showed typical intranuclear inclusion bodies, char-

acteristic of the virus infection, occurring in most of the parenchymal cells. Electron microscopy showed the presence of incomplete intranuclear and complete intracytoplasmic virus in the liver at the time of death.

Cellular entry of enveloped and deenveloped virus. Studies were undertaken to determine the mode of entry of enveloped and deenveloped EAV. It did not seem feasible to attempt to demonstrate attachment and penetration of particles in the *in vivo* system; thus, L-M cell monolayers were used to illustrate this process. Figures 8 to 11 reveal a viropexis sequence of partial or complete entry of enveloped virus within 5 min postinfection. In Fig. 8, 9, and 11, the virus is surrounded by pseudopodia. These elements appear to join (Fig. 9 and 11, arrows), forming fusion vacuoles containing from one to several particles (Fig. 10, 11).

A similar mechanism was observed with entry of deenveloped particles. Figures 12 to 14 show deenveloped virus free and in contact with the L-M cells surface membrane and in the process of entry at 10 and 15 min postinfection. The particles appear to enter by being phagocytized at the cell surface (Fig. 12 to 14), with the result that they become situated in intracytoplasmic vacuoles (Fig. 14, 15).

In Fig. 8 to 11, almost all particles appear to have intact envelopes, even after being phagocytized, which is the typical appearance for this time period. Generally speaking, few particles possessed broken envelopes and none was observed fusing with the plasma membrane. Fusion of the envelope is apparently not necessary for entry, as evidenced by the ability of deenveloped particles to enter cells (Fig. 14, 15) and by presence of intact envelopes surrounding free particles inside vacuoles.

After phagocytosis, virus of both types appeared to be largely localized to vacuoles, with few being recognized free in the cytoplasm up to 2 hr postinoculation of cells. At 12 to 24 hr, many vacuoles contained remnants of particles in all stages of dissolution as well as abundant, apparently intact virus. At 24 hr, the picture was complicated in cells receiving enveloped virus because particles were observed budding from the nucleus into the cytoplasm. There was no

FIG. 7. Radioactivity and density analysis of whole and deenveloped virus. Approximately equal amounts (5×10^{10} particles/ml) of ^3H -thymidine-labeled enveloped and deenveloped virus were mixed together and subjected to sucrose density gradient centrifugation. Peak 1 represents deenveloped virus; peak 2, normal virus. Conditions for centrifugation appear in the text.

TABLE 1. Determinations from whole blood, purified enveloped, and deenveloped virus

Viral prepn	LD ₅₀ /ml	No. of viral particles per LD ₅₀
Whole blood.....	$10^{9.70}$	3.3×10^2
Purified enveloped.....	$10^{5.60}$	3.6×10^6
Purified deenveloped.....	$10^{1.18}$	4.1×10^{10}

FIG. 8. Thin section of stage of entry of enveloped EAV into L-M cells by pseudopodia engulfment at 5 min postinfection. Stained with uranyl acetate and lead citrate. Particle being surrounded by pseudopodium. $\times 52,000$.

evidence of replication in cells receiving de-enveloped virus.

DISCUSSION

The ability of intact herpes virions to enter cells has been described as a viropexis or phagocytic mechanism whereby virions are engulfed at the cell surface and transported to the interior of the cell within a phagocytic vacuole (11, 13, 19). However, Morgan et al. (19) postulated that an alternative mechanism may exist, especially in relation to the infectious process. They suggest an enzyme-mediated event whereby, after virus attachment to the cell surface, disintegration of the envelope occurs in the area adjacent to the cell wall. The remnants of the envelope fuse with the cell wall, which is digested to allow passage of the capsid directly into the cytoplasm. The capsid is then digested, with subsequent release of the core. In the present study, the principle mechanism of EAV entry is the engulfment by pseudopodia which seem to either surround the virus and fuse with the cell membrane or fuse to another pseudopodium. This process results in the formation of intracytoplasmic vacuoles containing viral particles.

We could find no clear-cut evidence of virions whose envelopes were fused to the cell wall.

It is our experience that L-M cells grown in monolayer cultures exhibit numerous prominent pseudopodia, whereas they are inconspicuous in suspension cultures. This may explain why other investigators have not observed their activity in relation to this type of engulfment of viral particles. Also, the technique of fixation for electron microscopy in situ, eliminating preliminary washing, is important in order to not disturb the pseudopodia. It is also important to make observations very early after addition of virus to observe the earliest evidence of entry into the cell. Cultures fixed after 15 min may not show the dynamic events of phagocytosis and formation of vacuoles.

There is general consensus that enveloped particles of herpes virus are infectious; however, the role of the envelope has been the subject of debate. In a recent review by Darlington and Moss (6), the matter continues to remain in doubt. The efficiency of naturally occurring un-enveloped particles to infect was many times less than that of enveloped particles. It was

FIG. 9. Thin section of stage of entry of enveloped EAV into L-M cells by pseudopodia engulfment at 5 min postinfection. Stained with uranyl acetate and lead citrate. Particles being surrounded by two pseudopodia which appear to be in the process of fusing (arrow). $\times 50,000$.

FIG. 10. Thin section of stage of entry of enveloped EAV into L-M cells by pseudopodia engulfment at 5 min postinfection. Stained with uranyl acetate and lead citrate. Several particles present in intracytoplasmic vacuoles ostensibly due to fusion of pseudopodia to cell membrane. $\times 52,000$.

suggested that the envelope is involved in a specific attachment mechanism.

The data show that selective treatment of mature forms of EAV with the nonionic detergent, Nonidet P-40, and by sonic treatment has yielded clean preparations of deenveloped virus. It is apparent from the morphological evidence that this form closely resembles the incomplete or unenveloped naked virion present in the nuclei of infected cells (4, 20). Whether the mechanically prepared forms are identical to the naturally occurring unenveloped counterparts remains to be established.

The results show clearly that the deenveloped particles readily penetrate cells essentially by the process demonstrated for enveloped particles. It is evident that the viropexis mechanism is functional whether the particle is enveloped or not; furthermore, if the envelope is absent, fusion of this structure of the cell membrane is a priori excluded as the only mechanism of entry. It remains for future work to show the specificity of viropexis and the function of the envelope. It is apparent that the procedures used to remove the envelope yield poor-quality infectious virus which is more susceptible to deoxyribonuclease.

It also appears that deenveloped virus may be infectious for hamsters, although a prodigious number of particles is required to kill animals.

FIG. 11. Thin section of stage of entry of enveloped EAV into L-M cells by pseudopodia engulfment at 5 min postinfection. Stained with uranyl acetate and lead citrate. Virus present in fusion vacuoles. Arrow indicates possible area of fusion of one pseudopodium to another, resulting in particle being situated in vacuole. $\times 60,000$.

FIG. 12. Thin section of deenveloped virus entering L-M cell at 10 min postinfection. Particles are free and in contact with the cell membrane. Some appear to be in process of being engulfed by pseudopodia, whereas others are in a cytoplasmic invagination. Stained by uranyl acetate and lead citrate. $\times 30,000$.

FIG. 13. Deenveloped particle entering cell via engulfment by pseudopodia. Arrow indicates particle; 10 min postinfection. Stained by uranyl acetate and lead citrate. $\times 30,000$.

FIG. 14. Deenveloped EAV in contact with L-M cell surface membrane and present in an intracytoplasmic vacuole (arrow); 15 min postinfection. Stained by uranyl acetate and lead citrate. $\times 68,000$.

The infectivity in comparison to enveloped virus was reduced by more than 99.99%. It is pertinent to ask whether deenveloped particles are infectious, since their contamination by a few enveloped particles would escape detection by electron microscopy and could account for the residual infectivity. In an attempt to establish this point many grids were examined, with approximately 27,000 deenveloped particles being counted, and in no instance was an enveloped particle recognized. It is probably impossible

with the methodology available to absolutely rule out the possibility that a few enveloped particles survived the vigorous preparation and purification of the deenveloped form; however, it does not appear likely.

The analysis of isotopically labeled enveloped and deenveloped forms of EAV and the separated envelope are currently being investigated by polyacrylamide gel electrophoresis to establish their protein composition. Preliminary results show significant, marked differences.

FIG. 15. Several deenveloped particles present in an intracytoplasmic vacuole of L-M cell; 15 min post-infection. Stained by uranyl acetate and lead citrate. $\times 38,000$.

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