**Morphogenesis of the Assembly and Release of Bovine Enterovirus**

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**SUMMARY**

Fluorescent antibody (FA) studies of cells infected with bovine enterovirus showed cytoplasmic blebs with specific fluorescence to the virus. These structures were also found extracellularly in the debris of lysed cells and were RNA-positive by acridine orange (AO) staining. The morphology of virus-infected cells was further studied by scanning electron microscopy (SEM). Transmission electron microscopy (TEM) with immunoferritin tagging showed the development of long sacs with bilaminated and multilaminated membranes. These sacs had multiple twists at different intervals along their length forming a chain of vesicles. The development and maturation of the virus were observed in these vesicles. A number of virus-containing vesicles were also present extracellularly in the debris of lysed cells. In addition, virus was observed in layers of membranous cisternae closely associated with vacuoles and plasma membrane. Some of the cisternae opened to the extracellular space and appeared to allow the release of the virus. Virus particles were also found in patches and in crystals within the cytoplasmic matrix. Many lysed cells contained fibrils often associated with patches of ferritin-tagged virus. This study presents morphological evidence for the release of the virus in vesicles after cell lysis, via cisternae with openings to the extracellular space, and in cytoplasmic blebs.

Entry, assembly and release of a number of viruses in the family *Picornaviridae* have been studied to varying degrees by several investigators. Poliovirus has been shown to develop in membrane-enclosed bodies (Horne & Nagington, 1959), free in the cytoplasmic matrix and aligned along the elements of filamentous complexes (Dunnebacke et al. 1969). Poliovirus has also been shown to be released by way of a tubulovacuolar excretory septum, cytoplasmic fibrils and cytoplasmic blebs (Levinthal et al. 1969). Hackett (1961) and Zee et al. (1968) showed that cytoplasmic bubbling was a prominent feature of swine kidney cells (PK 13) infected with vesicular exanthema of swine virus (VESV). These cytoplasmic bubbles were also shown to contain RNA-positive granules by acridine orange (AO) staining and specific VESV antigen by fluorescent antibody (FA) staining. Recently, we showed the role of cytoplasmic blebs of infected cells in the prelytic release of foot-and-mouth disease virus (FMDV) (Yilma et al. 1978). In the present study, the morphogenesis of the assembly and release of bovine enterovirus (BEV) in bovine kidney cells is investigated by FA staining, AO staining, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) with immunoferritin tagging. In addition, the similarities and differences of the modes of assembly and release of BEV are compared with the studies done on other picornaviruses.

Except for a few changes noted, the methods used for these experiments were those used in previous work (Yilma et al. 1978). Secondary calf kidney cells originally obtained from gnotobiotic animals were used for virus propagation. The BEV used was isolated from oesophageal-pharyngeal fluid of a steer at the Plum Island Animal Disease Center and designated as BEV-PI-2. Cell cultures were first rinsed three times with Eagle's minimum essential medium (MEM) and inoculated with BEV at a multiplicity of 10. After 1 h of
adsorption at 37 °C, cells were rinsed twice with MEM to remove unadsorbed virus. Infected cell cultures were then incubated in MEM at 37 °C in a humidified chamber in 6% CO₂ for a designated length of time.

The BEV harvested from tissue culture fluid was concentrated 100-fold by two consecutive precipitations with 6% (w/v) polyethylene glycol. The virus was then purified by centrifugation on a discontinuous preformed CsCl density gradient for 150 min at 37,500 rev/min in a SW50.1 rotor (Trautman & Sutmoller, 1971). The visible light-scattering band at a density of 1.34 g/ml was mixed in equal parts with incomplete Freund's adjuvant to immunize rabbits subcutaneously. Three consecutive injections were administered at intervals of 10 days and serum was harvested 10 days after the last injection. The gamma globulin-enriched fraction of the antiserum was obtained by precipitation with ammonium sulphate, and its protein content was determined by the method of Lowry et al. (1951). The purified globulin fraction was directly conjugated to fluorescein isothiocyanate by the method of Clark & Shepard (1963). The direct method of FA staining technique was used in this study as described previously (Yilma et al. 1978). The conjugate showed specific fluorescence when tested on cultures infected with BEV but lacked such activity in cultures infected by a heterologous picornavirus, FMDV. Pre-treatment of samples with unlabelled anti-BEV serum blocked or severely diminished fluorescence when later stained with the conjugate. Similar treatment with normal serum lacked inhibitory activity against the conjugate.

Methods for AO staining, SEM, TEM and ferritin tagging were as previously cited (Yilma et al. 1978).

Immunofluorescent studies of the early development of BEV on coverslip cultures showed speckled, cytoplasmic fluorescence in fibroblastic cells. Blebs were formed and rounded cells released them 8 to 9 h p.i. The development of cytoplasmic blebs was also investigated by AO staining in parallel with the FA study. The results, except for a longer time interval, were comparable to those with FMDV (Yilma et al. 1978). The morphology of BEV-infected cells was further investigated by SEM. Results with SEM also showed knob-like structures in samples examined 10 h p.i. These structures were evenly distributed on the surface of rounded cells and gave the appearance of a berry-like structure. The micrographs were directly comparable to those with FMDV.

Results with TEM revealed several types of development of BEV in the cytoplasm of infected cells. The most common type observed in this study was the maturation of the virus in cytoplasmic vesicles. As early as 6 h p.i. long sacs were present with interlacing networks of bilaminated and multilaminated membranes. A series of twists observed in these sacs resulted in the formation of a chain of vesicles. These vesicles were either empty or filled to varying degrees with amorphous material that appeared to be the precursor for mature virus particles. At 16 h p.i., a number of vesicles were observed containing crystalline arrays of virus particles (Fig. 1a, b). These particles had an average diam. of 25 nm, which is the size of BEV (Andrewes & Pereira, 1972). In general, these vesicles had diam. ranging from 100 to 300 nm. This variability in diameter may not only be related to actual size differences alone but could also be due to tangential sectioning of the vesicles during specimen preparation. A large number of intact, cytoplasmic vesicles were also present extracellularly in the debris of lysed cells (Fig. 1c). These cytoplasmic vesicles may serve as one possible release mechanism for BEV from infected cells. Only virus particles located on the surface of these vesicles, but not internally, were accessible to tagging with the ferritin conjugate (Fig. 1d).

Virus particles were also observed in several layers of membranous cisternae closely communicating with a series of cytoplasmic vacuoles (Fig. 2a). The membranes of these cisternae appeared to be branching off from the double membrane of the vacuoles and had an internal diam. of 30 to 35 nm. Each cisterna contained a single row of virus particles
Fig. 1. Transmission electron micrographs of cells infected with BEV. (a) Bilaminated and multilaminated cytoplasmic vesicles 16 h p.i. Note that the vesicle containing the virus crystal is a part of a long sac twisted at least five sites (arrows) to form a series of vesicles. (b) Tracing of a chain of vesicles in (a). (c) Two extracellularly located virus-containing vesicles (arrows) in the debris of lysed cells 14 h p.i. (d) Virus-containing vesicle in the debris of lysed cells 18 h p.i. Note that the four virus particles on the surface of the vesicle (arrow) are tagged with the ferritin conjugate.
Fig. 2. Transmission electron micrographs of cells infected with BEV. (a) Virus particles in membranous cisternae connected to empty vacuoles 14 h p.i. (b) A membranous cisterna with openings to the extracellular space 16 h p.i. Note that virus particles (arrow) appear to be released to the extracellular space. (c) Cytoplasmic fibrils with a patch of virus particles in the extracellular space 18 h p.i. Note that virus particles are grouped together, probably as the result of interconnexion with the ferritin conjugate.
with an average diam. of 25 nm. None of the vacuoles associated with these cisternae observed in this study contained any virus particles. A number of these membranous cisternae were closely associated with the plasma membrane and appeared to allow the release of virus to the extracellular space (Fig. 2b). Frequently, the debris of lysed cells contained cytoplasmic fibrils invariably associated with free virus particles (Fig. 2c).

The different sites where virus was found were not limited to intracytoplasmic vesicles and membranous cisternae. In addition, in a pattern very similar to that of FMDV (Breese & Graves, 1966; Breese, 1969), patches of free virus were observed within the cytoplasmic matrix or developing into a crystalline array. Virus particles were also found in free and cell-associated cytoplasmic blebs. Cytoplasmic blebs may serve as one of the mechanisms by which BEV is released from infected cells. Similarly, cytoplasmic blebs have been shown by FA and AO staining in cells infected with VESV (Zee et al. 1967), poliovirus (Mayor, 1961) and FMDV (Yilma et al. 1978).

Results with TEM showed that BEV assembly took place predominantly inside cytoplasmic vesicles with bilaminated and multilaminated membranes with diam. of 100 and 300 nm. The formation of a chain of vesicles by a series of twists on a long sac with bilaminated and multilaminated membranes (Fig. 1a) in virus-infected cultures is reported for the first time in this study. These sacs are probably virus-coded because they begin to appear 6 h.p.i. and since cellular protein synthesis is shut down early in picornavirus-infected cells (Zimmerman et al. 1963). In addition, the absence of these sacs in uninfected, control cultures and their presence in BEV-infected cultures would suggest that they are virus-coded. Dales et al. (1965) described bodies limited by a single membrane and 70 to 200 nm in diam. containing one or more virus particles in poliovirus-infected HeLa cells. Penman et al. (1964), from their studies with gradient sedimentation of homogenates from poliovirus-infected cells, concluded that poliovirus was synthesized within membrane-bound structures. Dunnebacke et al. (1969) also described small, round cytoplasmic vesicles in poliovirus-infected HeLa and human chorion cells, often enclosing a single particle. In contrast, vesicles observed in BEV-infected cells for the most part contained large numbers of virus particles.

A number of free virus particles were observed emerging from plasma membranes. These virus particles were easily distinguished from cellular ribosomes because they were easily accessible for ferritin antibody tagging. In general, mechanisms of release of BEV are several and similar to those of FMDV (Yilma et al. 1978).

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