Morphogenesis of Berne Virus (Proposed Family Toroviridae)

By MARIANNE WEISS1* AND MARIAN C. HORZINEK2

1Virology Department, Institute of Bacteriology, Veterinary Faculty, University of Berne, Switzerland and 2Institute of Virology, Veterinary Faculty, State University Utrecht, Yalelaan 1, 3508 TD Utrecht, The Netherlands

(Accepted 26 February 1986)

SUMMARY

In equine dermis cells infected with Berne virus particles were first detected 10 h after infection. Virions were encountered in all parts of the Golgi system and, infrequently, in the rough endoplasmic reticulum. A unique form of budding of pre-assembled rigid tubular nucleocapsids was demonstrated. Masses of tubular nucleocapsids of a lesser diameter and electron density were prominent in the cytoplasm and the nucleus of infected cells. Within the Golgi system and cytoplasmic cisternae virions appeared as straight or slightly curved rods. Extremely long, aberrant virions (250 nm) were occasionally seen. The proper torovirion morphology was observed in extracellular particles and in vacuoles near the cell surface.

INTRODUCTION

In 1972 a virus was isolated during routine laboratory diagnostic work from a horse under observation at the Surgery Clinic in Berne, Switzerland. This P138/72 strain of Berne virus is the only isolation in vitro so far of a new group of viruses, for which family status has recently been proposed (Horzinek & Weiss, 1984). Other members of this taxonomic cluster are the Breda virus serotypes characterized by Woode et al. (1982) and Saif et al. (1981). These viruses, however, have to be propagated by calf-to-calf passages and cannot be grown in culture.

Purified Berne virions contain two major proteins with mol. wt. of 20000 (20K) and 22K. The 20K polypeptide is phosphorylated and possesses RNA-binding properties; it is therefore considered to be the main capsid protein (Horzinek et al., 1985). The other major protein of 22K is non-glycosylated and membrane-associated. Together with a 37K phosphoprotein, these polypeptides may function as matrix proteins. Two glycosylated polypeptides of 120K and 80K are probably subunits of the peplomers of the virion (M. C. Horzinek et al., unpublished observations). The genome of Berne virus is a single-stranded, colinear molecule of about 6 x 106 mol. wt. and is of messenger polarity. In infected cells, six subgenomic polyadenylated RNAs probably possessing messenger function have been identified (M. C. Horzinek et al., unpublished results).

Torovirions are pleomorphic particles having a spherical, oval, elongated or kidney-shaped morphology, and measuring 120 to 140 nm in diameter. The morphological data are best explained by assuming a helical nucleocapsid tightly coiled into a hollow tube which is either straight or bent into an open torus. A tightly fitting envelope surrounds this structure. Consequently, the virion assumes a crescent or kidney shape with the membrane following the smaller curvature of the capsid or enveloping the whole torus, conferring an erythrocyte-like morphology to the particle (biconcave disk). Thin-section electron micrographs support these observations (Weiss et al., 1983).

The present paper reports the morphopoietic events which result in release of mature Berne virus particles from infected cells. The unique appearance of preformed tubular nucleocapsids is reported; these are enveloped during a budding process into Golgi vesicles and intracytoplasmic cisternae.
METHODS

Virus and cell culture. Berne virus (strain P138/72) was propagated in equine dermal cells grown in Eagle's MEM and supplemented with non-essential amino acids (1%), L-glutamine (200 mM), sodium bicarbonate, antibiotics and 2 to 10% foetal calf serum (FCS) which had been prescreened for the absence of antibody against toroviruses by neutralization tests (Weiss et al., 1984). Cells were regularly checked for mycoplasma contamination by chemiluminescence (Bertoni et al., 1985) and electron microscopy. Virus stock solutions (cell culture supernatants after low-speed centrifugation) had been treated with α-phenylethanol (Merck, Darmstadt, F.R.G.; Staal & Rowe, 1974) and tested in mycoplasma broth (Bannermann & Nicolet, 1971) to ascertain absence of these organisms.

Infectivity assay. Serial tenfold dilutions of the virus preparations were added to flat-bottomed microtitre plates (Greiner & Söhne, Nürtingen, F.R.G.) in 100 µl volumes containing monolayers of 3 × 10⁴ cells/well. The Spearman–Kärber formula was applied for the calculation of infectivity titres (ID₅₀) after reading cytopathic effects 5 days after infection.

Preparation for electron microscopy. Confluent monolayers of equine dermal cells in 25 cm² plastic flasks (Nunc) were rinsed once with phosphate-buffered saline (PBS) and inoculated with Berne virus at a multiplicity of 0.2 to 2 ID₅₀/cell (time 0). One h later the inoculum was replaced by cell culture medium after one rinse with PBS. Mock-infected cultures served as controls.

Fixation and embedding. At different times after inoculation 100 µl samples of the cell culture supernatants were harvested for determination of virus yields and the monolayers were prepared for thin-section electron microscopy. For this purpose cells were removed from the support by a short trypsinization [trypsin (Difco) 1:250, 0.05%, 0.5 mM-EDTA in PBS, pH 7.2] and suspended in cold 2.5% glutaraldehyde (EM grade; Electron Microscopy Sciences, Fort Washington, Pa., U.S.A.) in 0.125 M-sodium cacodylate buffer (dimethylarsinic acid sodium salt, trihydrate; Merck-Schuchardt, Hohenbrunn, F.R.G.), pH 7.4. After incubation for 2 h at 4°C the samples were washed in cacodylate buffer and postfixed for 1 h in 1% osmium tetroxide (EM grade, purified; Electron Microscopy Sciences) in the same buffer.

In some experiments, cells were fixed in situ with glutaraldehyde, then rinsed briefly with cacodylate buffer and removed from the support with the help of a rubber policeman into fixation buffer containing 1% osmium tetroxide. Postfixation was followed by several rinses in cacodylate buffer and presoaking in a solution of 2% uranyl acetate (pro analysi; Merck) in 70% acetone. The cells were sedimented by centrifugation, dehydrated in an ascending series of acetone concentrations followed by 1,2-propylene oxide (Merck) and finally embedded in resin [glycid ether 100 (Merck), nadic methyl anhydride (Acima, Buchs, Switzerland), dodecenylsuccinic anhydride (Acima), 2,4,6-tris(dimethylaminomethyl)phenol, (Merck)].

Ultrathin sections were prepared with the help of a Reichert microtome and were stained with lead citrate (Venable & Coggeshall, 1965) and examined in a Philips EM 300 electron microscope operating at 80 kV.

RESULTS

The growth kinetics of Berne virus in equine dermal cells is shown in Fig. 1. Characteristically there was an increase in extracellular activity starting between the 8th and 9th h after infection, reaching a plateau at about 15 h. Cytopathological changes were noticeable only about 21 h after infection in 10% of the cells. By electron microscopy the first virus particles were detected at about 10 h post-infection.

For up to 6 h after infection, preparations made from the infected samples were indistinguishable by electron microscopy from the uninfected controls. No morphological evidence of a virus infection was obtained. Between 7 and 9 h after infection large polysomal aggregates were encountered free in the cytoplasm. At that time infected and mock-infected cultures showed signs of high synthetic activity: the cisternae of the rough endoplasmic reticulum were distended, with many membrane-bound ribosomes and free ribosomes arranged in groups. The large polysomes were preferentially found near the cytoplasmic surface and the nuclear membrane. Although no quantification was made, the impression was gained that these structures were more abundant and larger in infected cultures (Fig. 2).

About 10 h after infection virus particles were first seen within parts of the unaltered Golgi apparatus (Fig. 3), and also extracellularly (not shown). At that time tubular structures (tubuli) were visible in the cytoplasm and in the nucleus of infected cells. Their appearance in both compartments was morphologically identical. They were of variable length, electron density,
Morphogenesis of Berne virus

Fig. 1. Kinetics of virus growth in Berne virus-infected equine dermis cells. Arrow indicates time of appearance of enveloped virus particles in electron microscopic thin sections.

Fig. 2. Giant polysomes free in the cytoplasm near the nuclear membrane. N, Nucleus. Bar marker represents 100 nm.

Fig. 3. Berne virus-infected cell 10 h after infection. Enveloped virus particles (arrows) are seen within Golgi cisternae. Bar marker represents 100 nm.

and of diameter varying along an individual strand between 10 and 32 nm; most were in the 14 to 27 nm diameter range. In the nucleus tubuli appeared either singly or in densely packed aggregates (Fig. 4). These structures were never observed entering or leaving the nucleus.

In the cytoplasm the tubular structures were encountered singly (Fig. 5b) or in small groups (Fig. 5a), rarely in larger accumulations (see Fig. 18). They were frequently observed in the immediate proximity of electron-dense granular material, some of which showed ring-like cross-sections and short elongated transitions to tubuli (Fig. 5a). Regions of fine filaments similar to viroplasm 'factories' were also seen in the infected cells (Fig. 6).

Budding of virus particles singly or in groups was observed predominantly in the Golgi system (Fig. 7). From the images obtained at different stages of the process the following sequence of events can be reconstructed. The preformed intracytoplasmic nucleocapsid approaches the
membrane with one of its rounded poles (Fig. 8a) upon which the membrane progressively attaches to the capsid along one side (Fig. 8b). It should be noted that the part of the nucleocapsid which is already enveloped has a higher electron density and a uniform diameter whereas the part still embedded in the cytoplasmic matrix is of lesser electron density and varying width, as can be clearly seen in Fig. 8(c). The result of this morphopoietic process is an entirely enveloped tubulus which is encountered free in the lumen of the Golgi cisterna. The particles are without exception rod-like with two rounded ends and a regularly structured core of uniform electron density (Fig. 9). Their bacilliform structure is emphasized by occasional packaging into bundles (Fig. 10). In cross-sections, the virion appears as three concentric circles of high electron density; the innermost circle of highest electron density probably constitutes a transverse section through the nucleocapsid. Its electron-lucent centre indicates the tubular nature of the capsid (Fig. 11). The diameters of the circles are 24 nm (n = 9), 37 nm (n = 7), and 47 nm (n = 8), respectively.

As mentioned above, virion morphogenesis is mainly observed in the Golgi system, in the lateral vesicles as well as the flat cisternae. Sometimes virions can be observed in several cisternae of the same stack (Fig. 12). Late in infection, budding occurs also into smooth-surfaced vesicles in locations distant from the nucleus, where the correlation to the Golgi system can no longer be established. Additional budding into the rough endoplasmic reticulum (Fig. 13) and the perinuclear space (Fig. 14) has occasionally been observed.

Virus-containing vesicles which may be coated (Fig. 15) or not merge with the peripheral plasma membrane and release their contents. Virus is then found accumulating at the peripheral cytoplasmic membrane and shows the twin circular structures characteristic of torovirion morphology (Fig. 16). Aberrant virion forms of extreme length (Fig. 17) are occasionally observed in vacuoles; their length can exceed 250 nm.

At the time of the first appearance of virions in infected cells (about 10 h post-infection) the cells appear normal; later on, the Golgi system may appear dilated and conspicuous aggregates of vesicles, sometimes with embedded nucleocapsids, can be observed (Fig. 18). Degenerative processes involving the mitochondria and the nucleus can be seen as well as virions enclosed in autophagic vacuoles (Fig. 19).

**DISCUSSION**

Following our description of Berne virions in negatively stained and thin-sectioned preparations (Weiss et al., 1983) and similar data presented for Breda virus by Woode and his collaborators (Woode et al., 1982; Pohlenz et al., 1985; Fagerland et al., 1986) we here present data on the morphopoiesis of a torovirus. Budding has been observed at intracytoplasmic membranes, in most cases membranes of the Golgi system. This is not unlike the situation in other enveloped RNA viruses, e.g. bunya- (Murphy et al., 1968; Smith & Pifat, 1982), corona- (Takeuchi et al., 1976; Massalski et al., 1981; Dubois-Dalcq et al., 1982) and rhabdoviruses (Murphy & Harrison, 1979; Fekadu et al., 1982). However, Berne virus displays unique features of budding: a rigid, tubular capsid which has been preformed at a site different from the place of budding is incorporated into the virion. Preformed nucleocapsids are also encountered in cells infected with paramyxoviruses which, however, show a different morphogenesis (budding at the peripheral cytoplasmic membrane, sideways attachment of the capsid to the membrane; Wolinsky et al., 1974; Dubois-Dalcq et al., 1984). Another important feature of Berne virus morphogenesis is a morphological change which the capsid undergoes during budding. We have the impression that the irregularly coiled, electron-lucent nucleocapsid becomes progressively

---

**Fig. 4 and 5.** Tubular structures in nucleus and cytoplasm. In both compartments, the electron density and diameter of the tubuli vary along the strands. Fig. 5. (b) Tubuli scattered in the cytoplasm (arrowheads; arrow, budding virion). A microtubule is indicated by stars. (a) Aggregations of electron-dense granular material and a tubular structure (large arrowhead) in its immediate proximity are demonstrated. The small arrowhead points to a cross-section and the arrows to short elongated transitions to tubuli. Bar markers represent 100 nm.

**Fig. 6.** Intracytoplasmic inclusion in Berne virus-infected cell consisting of irregularly dispersed filamentous material. Bar marker represents 100 nm.
Fig. 7. Viruses budding from the membranes of Golgi cisternae (arrowheads). Arrow indicates an enveloped particle in a smooth-surfaced vesicle. Bar marker represents 100 nm.

Fig. 8. Various stages of viral budding into smooth membrane vesicles. (a) Initial stage: one pole of the nucleocapsid is fixed to the membrane (arrowhead). (b) Membrane attached to one side of the capsid (arrowhead). (c) Enveloped part of the nucleocapsid protruding into the vesicular lumen; it possesses a uniformly high electron density and uniform diameter. The other end of the capsid which is still situated in the cytoplasmic matrix is of lesser electron density and of varying width. Bar markers represent 100 nm.

Fig. 9. Enveloped bacilliform virus particle within a smooth membrane structure. Bar marker represents 100 nm.

Fig. 10. Bundles of rod-shaped virions in the lumen of smooth surfaced cytoplasmic cisternae. Bar marker represents 100 nm.

Fig. 11. Cross-section through an enveloped virion. The electron-lucent centre represents the (hollow?) inner space of the tubular nucleocapsid which itself appears as the electron-dense inner ring. The nucleocapsid is surrounded by a moderately dense intermediate and an electron-lucent outer zone delineated by a dark contour. Bar marker represents 25 nm.
Morphogenesis of Berne virus

Fig. 12. Virus particles present in different cisternae of a Golgi stack. Bar marker represents 100 nm.

Fig. 13. Virus particle budding into a cisterna of the rough endoplasmic reticulum. Bar marker represents 100 nm.

Fig. 14. Virus particle budding into the perinuclear space (part of the nucleoplasm is shown in the left upper half). Bar marker represents 100 nm.

Fig. 15. Virus particles being released from the cytoplasm membrane by exocytosis. One particle of bacilliform shape is contained in a coated vesicle near the cell surface whereas another virion shows the curved morphology characteristic of extracellular Berne virus. Arrowhead points to a twin circular virus structure in the extracellular space (cross-section through a torus). Bar marker represents 100 nm.

straight and electron-dense as it becomes enveloped. It can be speculated that this is due to the interaction of the nucleoprotein with a putative matrix polypeptide. This is in contrast to the situation with rhabdoviruses, where budding is coordinated with coiling of a helical nucleoprotein strand into a tubular nucleocapsid structure (Odenwald et al., 1984).

It has been mentioned that within the cytoplasmic vesicles and cisternae virions have a rod-like shape. Those particles, however, which have been released or are about to be released possess the characteristic torus form. Consequently, it has to be postulated that further morphopoietic events occur during the transition of intravesicular virions to the extracellular state. These observations are supported by data presented on Breda virus morphology where bacilliform viruses have been encountered in thin sections through infected enterocytes.
Fig. 16. Released Berne virus particles adsorbed to the plasma membrane of an infected cell. The twin circular structures represent cross-sections through the toroidal tubular nucleocapsids. The defined distance of the virions from the plasma membrane indicates the presence of peplomers. Bar marker represents 100 nm.

Fig. 17. Virion of extreme length (253 nm) in a cytoplasmic vesicle. Bar marker represents 100 nm.

Fig. 18. Advanced stage of virus infection: accumulations of nucleocapsids (arrows) between aggregates of vesicles. Enveloped virions (arrowheads) are contained in different kinds of cytoplasmic cisternae. Bar marker represents 500 nm.

Fig. 19. Virus particles enclosed in a lysosome-like body. Bar marker represents 100 nm.

(Pohlenz et al., 1985; Fagerland et al., 1986) whereas torovirions of the characteristic shape are prevalent in faecal samples (Woode et al., 1982). Consequently, the biconcave disk torovirion morphology which we have described cannot be considered an artefact that has arisen during in vitro cultivation of Berne virus.

From the observation that virions are encountered mostly in vesicles derived from the Golgi system it can be assumed that they make use of the normal cellular excretory pathway, not unlike coronaviruses (Sturman & Holmes, 1983). This would explain the long period of virus excretion and the late onset of cytopathic effect.

Viral capsids have been encountered in the nucleus; it is not known whether this represents a dead-end stage in virion assembly or is connected with the dependence of virus replication on nuclear functions as described before (Horzinek et al., 1984).
Dependence of Berne virus replication upon some nuclear function of the host cell implies that sequential studies of the virion morphogenesis must be made in synchronized cells. Indeed, even at very high multiplicities of infection only a small fraction of cells was found by electron microscopy to contain virions. The essence of budding, however, is unlikely to be different under synchronous conditions from the observations presented here, i.e. attachment of a preformed capsid by one end to the cytoplasmic face of a Golgi membrane, sideways attachment of the membrane to the capsid and progressive extrusion with final detachment and release into the vesicular lumen.

The authors should like to thank Marlies Koller for preparing the thin sections.

REFERENCES


*(Received 2 January 1986)*