Replication and Release of Epizootic Haemorrhagic Disease Virus in BHK-21 Cells

By R. Lewanczuk and T. Yamamoto*

Department of Microbiology, University of Alberta, Edmonton, Alberta, Canada T6G 2E9

(Accepted 4 March 1982)

SUMMARY

Epizootic haemorrhagic disease virus (EHDV) was seen by light and electron microscopy to replicate in perinuclear locations. Tubules, paracrystals and virus matrices were associated with replication sites. As infection proceeded, aggregates of virus migrated towards the cell periphery, resulting in cell membrane rupture near the virus aggregate with the subsequent release of the virus aggregates. Virus release, as seen by light microscopy, gave the appearance of occurring by a 'budding' process whereby part of the cell would swell and subsequently rupture or break away. Infectivity studies indicated that approx. 80% of newly replicated virus was released extracellularly in aggregates which required disruption to maximize infectious virus yield. Trypsin did not enhance virus infectivity. Of the six EHDV isolates used in this study each isolate was characterized by its own maximum yield obtained after several serial passages in cell culture.

INTRODUCTION

Epizootic haemorrhagic disease virus (EHDV) is an orbivirus of the Reoviridae family and is classified in the bluetongue subgroup (Joklik, 1974). It is known to replicate in cytoplasmic matrices in susceptible cells (Tsai & Karstad, 1970; Murphy et al., 1971; Thomas & Miller, 1971; Tsai & Karstad, 1973). Infection is associated with tubule formation in the infected cells (Tsai & Karstad, 1970, 1973; Murphy et al., 1971; Thomas & Miller, 1971; Verwoerd et al., 1979). Infected cells stained by the fluorescent antibody or acridine orange techniques display cytoplasmic inclusions which are usually perinuclear in position (Jochim et al., 1974; Bando, 1975; Fosberg et al., 1977). Upon maturation, release of EHDV from cells has been reported to occur through cell lysis (Tsai & Karstad, 1970; Murphy et al., 1971).

This study was carried out in order to investigate the observation that sonication of infected cell culture supernatant significantly increased infectious virus recovery to levels which surpassed those of cell-associated fractions. We have previously assumed that most virus remained cell-associated and had, as others (Tsai & Karstad, 1970, 1973; Murphy et al., 1971; Kontor & Welch, 1976; Huismans et al., 1979), used the infected cell fraction as an initial source of EHDV for purification. This finding, as well as the varying virus yields we had been obtaining under various conditions, prompted us to carry out a complete study of the events involved in EHDV replication and release.

METHODS

Cells and virus. BHK-21 cells were propagated as monolayers at 37 °C in Eagle's minimal essential medium (MEM) supplemented with 0.22% NaHCO₃, penicillin (100 IU/ml), streptomycin (100 µg/ml) and 5% heat-inactivated foetal bovine serum. Six EHDV isolates were used in this study. EHDV-Kentucky (EHDV-Ky) and EHDV-New Jersey (EHDV-NJ)
were isolated and passaged as described previously (Foster et al., 1977) and were each thereafter serially passaged three times in BHK-21 cells. EHDV-Charmony (EHDV-Charm) was isolated from a North Dakota deer in 1970, cloned in L-929 cells and grown in BHK-21 cells. The virus was then inoculated into a 2-year-old white-tailed deer (Odocoileus virginianus) which died 2 days after inoculation. The spleen was harvested and virus reisolated by one passage in BHK-21 cells (T. M. Yuill, personal communication). EHDV-North Dakota (EHDV-ND) was isolated from the liver of an infected deer in 1962, cloned in L-929 cells and grown in BHK-21 cells. EHDV-Wisconsin (EHDV-Wis) was derived from EHDV-NJ by passage in suckling mice and cell culture. The American Type Culture Collection strain of EHDV (EHDV-ATCC) was derived from EHDV-NJ by passage in suckling mice and cell culture. EHDV-NJ, EHDV-Wis and EHDV-ATCC represent serotype I EHDV. EHDV-ND, EHDV-Ky and EHDV-Charm represent serotype II EHDV. EHDV-NJ, EHDV-Ky and EHDV-Charm were provided by Dr T. M. Yuill, Department of Veterinary Science, University of Wisconsin, Madison, Wis., U.S.A. EHDV-ND and EHDV-Wis were provided by Dr F. C. Thomas, Animal Disease Research Institute, Ottawa, Canada. EHDV-ATCC was obtained from the American Type Culture Collection, Rockville, Md., U.S.A. Unless otherwise indicated EHDV-ATCC was used as the virus inoculum in this study.

Infectivity assay. Virus infectivity was quantified by a plaque assay. Growth medium was removed from BHK-21 cell monolayers in six-well tissue culture dishes and 0.1 ml amounts of 10-fold virus dilutions added. The virus was allowed to adsorb at 37 °C for 1 h after which 3 ml of overlay was added to each well. The composition of the overlay was as follows: 10% foetal calf serum, 10% tryptose phosphate, 0.3% sodium bicarbonate, 0.6% agarose (FMC Corporation, Rockland, Md., U.S.A.) all in L-15 (Liebovitz) medium (Difco). The infected monolayers were incubated at 37 °C in 5% CO₂ for 5 days after which they were fixed in 99% methanol and the overlay removed. Monolayers were stained with 0.9% crystal violet in 7% citric acid and plaques counted.

Location of infectious virus. BHK-21 cell monolayers were infected with the EHDV strains at a multiplicity of infection of 1. Monolayers showing greater than 90% cytopathic effect were centrifuged at 4080 g for 20 min and the cell and supernatant fractions separated. These fractions were then sonicated in 10 ml amounts for 45 s at maximum setting using the 3/8 inch diam. probe of a Biosonik III sonicator (Bronwill Scientific, Rochester, N.Y., U.S.A.). This approach resulted in maximum virus yield. Plaque assays were carried out on all fractions.

One-step growth curve. Cell monolayers containing a known number of cells were infected at a multiplicity of infection of 5. Adsorption was carried out at 37 °C for 1 h after which the monolayers were washed three times with MEM. Five ml MEM was added and the monolayers incubated at 37 °C in 5% CO₂. At appropriate intervals the supernatant was removed for separate assay. The cells cooled to 0 °C in a waterbath were resuspended in 5 ml MEM, centrifuged at 3000 g for 10 min and resuspended again in 5 ml MEM. The supernatant fractions were sonicated as described above and assayed for cell-free and cell-associated virus.

Effect of trypsin on EHDV infectivity. Known amounts of EHDV were treated with 5 µg/ml and 75 µg/ml trypsin according to the method of Svehag et al. (1966). EHDV-infected cell cultures were also treated with 5 µg/ml trypsin according to the method of Almeida et al. (1978). The treated samples were assayed for infectivity by plaque assay.

Cytological staining. Infected and uninfected BHK-21 cells grown on coverslips were fixed overnight with Carnoy’s fixative (ethanol: chloroform : acetic acid, 6:3:1) and rinsed with tris buffer pH 7.8. Acridine orange staining was carried out according to the method of von Bertalanffy et al. (1958). Slides were examined by reflected light fluorescence microscopy.
using a mercury vapour lamp and appropriate filters to produce a wavelength of 450 nm. Haematoxylin–eosin staining was carried out using Harris' haematoxylin (Humason, 1962).

**Preparation of EHDV antibodies.** An EHDV-infected BHK-21 cell culture was harvested, homogenized and centrifuged at 16300 g for 15 min. The supernatant was sonicated and used as an EHDV antigen source. The virus preparation, in vol. of 0.1 ml, was injected intravenously into New Zealand white rabbits weekly for 6 weeks. Following this treatment the virus suspension, mixed with an equal volume of Freund's adjuvant (total vol. 1-5 ml), was injected intramuscularly into the same rabbit at weekly intervals for 3 weeks. Rabbits were bled 20 days after the last injection by cardiac puncture. The blood was pooled, allowed to clot, the serum removed and centrifuged at 1000 g for 10 min. The serum was then incubated at 37 °C for 1 h with an equal volume of BHK-21 cell homogenate. The mixture was centrifuged at 16300 g for 15 min and the supernatant used as the virus antibody source.

**Fluorescent antibody staining.** An indirect fluorescent antibody staining technique was used in this study. Infected and non-infected BHK-21 cells on coverslips were fixed in acetone, rinsed in tris buffer pH 7.8 and 0.5 ml of the EHDV antiserum added. The coverslips were incubated at 37 °C for 30 min, rinsed with tris buffer and fluorescein-labelled goat anti-rabbit serum (Difco) added and allowed to adsorb for 30 min at 37 °C. The stained monolayer was then rinsed with tris buffer and examined with a reflected light fluorescent microscope using a mercury vapour lamp and appropriate filters to produce a wavelength of 500 nm.

**Electron microscopy.** EHDV-infected BHK-21 cells were harvested and centrifuged at 4080 g for 20 min. The supernatant was decanted and the cell pellet fixed in 3% glutaraldehyde for 3 h, washed four times in a 0-1 m-phosphate buffer with 10% sucrose (pH 7.6) and post-fixed in 2% phosphate-buffered osmium tetroxide (pH 7.2) for 3 h. Cells were then progressively dehydrated in ethanol and embedded in Epon 812 (Fisher Scientific, Forest Lawn, N.J., U.S.A.). Sections were cut using diamond knives, stained with uranyl acetate and lead citrate, placed on 400-mesh grids and examined using a Philips EM 300 electron microscope. Virus in supernatant fractions was examined after high-speed centrifugation (16300 g for 30 min) of supernatant fractions and preparation of the resulting pellet in the manner described for thin-section electron microscopy. Examination of sonicated and unsonicated supernatant-associated virus was carried out by negative staining electron microscopy after staining the respective fractions with 3% sodium phosphotungstate buffered at pH 7.6.

**RESULTS**

**Recovery of infectious virus**

Sonicated and unsonicated cell and supernatant fractions from infected cell cultures were assayed for infectious virus. Typical results are presented in Table 1 from which it is evident that sonication of culture supernatant was necessary in order to unmask full virus infectivity. After sonication it is apparent that extracellular virus comprised slightly over 80% of total infectious virus. Had the cells (but not the supernatant) been disrupted, misleading values of approx. 97% intracellular virus would have been obtained.

**Yield of various EHDV isolates**

The six EHDV isolates, when received, produced low infectious titres in BHK-21 cells. Additionally, titres of the various isolates varied considerably. With continued serial passage, however, virus titres were observed to increase to a maximum which was then maintained. Titres of all six EHDV isolates after six and 15 BHK-21 cell passages are given in Fig. 1.
R. LEWAN CZUK AND T. YAMAMOTO

**Fig. 1.** Effect of serial passage on infectivity yields of EHDV isolates. A known number of BHK-21 cells in monolayer were infected at a multiplicity of infection of 1 to 2. After the 6th (□) and 15th (■) passages the cultures were harvested and assayed for total infectious virus after sonication.

**Fig. 2.** One-step growth of EHDV-ATCC in BHK-21 cells. Cells were infected at a multiplicity of infection of approx. 5. Cells and supernatant were harvested and assayed as described in Methods.

- ■, Cell-associated virus; ● – ●, cell free virus.

### Table 1. Recovery of EHDV from infected BHK-21 cultures

<table>
<thead>
<tr>
<th>Culture fraction</th>
<th>Total virus (p.f.u.)</th>
<th>Total virus (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unsonicated cells</td>
<td>$4.0 \times 10^4$</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Sonicated cells</td>
<td>$1.4 \times 10^5$</td>
<td>18</td>
</tr>
<tr>
<td>Unsonicated supernatant</td>
<td>$4.2 \times 10^7$</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Sonicated supernatant</td>
<td>$6.4 \times 10^7$</td>
<td>82</td>
</tr>
<tr>
<td>Total sonicated fractions</td>
<td>$7.8 \times 10^7$</td>
<td>100</td>
</tr>
</tbody>
</table>

*Total virus = sonicated cells + sonicated supernatant.

### Table 2. Virus yields and release of EHDV isolates infecting BHK-21 cells

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Yield (p.f.u./cell)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Supernatant</td>
<td>Cell</td>
</tr>
<tr>
<td>ATCC</td>
<td>340</td>
<td>92</td>
</tr>
<tr>
<td>Charm</td>
<td>200</td>
<td>36</td>
</tr>
<tr>
<td>Ky</td>
<td>240</td>
<td>61</td>
</tr>
<tr>
<td>ND</td>
<td>160</td>
<td>40</td>
</tr>
<tr>
<td>NJ</td>
<td>120</td>
<td>22</td>
</tr>
<tr>
<td>Wis</td>
<td>250</td>
<td>55</td>
</tr>
</tbody>
</table>

*See text for explanation of isolates. The experiment was carried out as in Fig. 1. Results are after the 15th passage of the isolates.

Additionally, distribution of virus between cells and culture supernatant for all six strains after the 15th passage are given in Table 2. It is apparent from these results that with continued cell passage the titre of all EHDV strains could be raised to between 100 and 500 p.f.u./cell.
Fig. 3. EHDV-infected BHK-21 cell monolayer 12 h post-infection stained with haematoxylin–eosin. The arrows indicate the cytoplasmic inclusions.

Table 3. Effect of trypsin on EHDV infectivity

<table>
<thead>
<tr>
<th>Test sample</th>
<th>Trypsin amount (μg/ml)</th>
<th>Yield (p.f.u./ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purified virus*</td>
<td>0</td>
<td>$1 \times 10^7$</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>$1.5 \times 10^3$</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>0</td>
</tr>
<tr>
<td>Culture supernatant</td>
<td>0</td>
<td>$4 \times 10^6$</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>$2.5 \times 10^3$</td>
</tr>
</tbody>
</table>

* Virus was purified by polyethylene glycol precipitation and two CsCl isopycnic centrifugations and re-suspended at $1 \times 10^7$ p.f.u./ml.

One-step growth curve

A one-step growth curve of EHDV-ATCC is presented in Fig. 2. This curve shows that replication of EHDV-ATCC was complete at approx. 15 h and that release occurred thereafter until approx. 32 h at which time maximum levels of extracellular virus were achieved. At this latter time 350 p.f.u./cell had been released from the cells representing 78% of the infectious virus yield and 100 p.f.u./cell were cell-associated representing 22% of the infectious virus yield. The virus yield for EHDV-ATCC was thus approx. 450 p.f.u./cell. The eclipse period was about 5 h. Continuation of the experiment to 48 h indicated a slight decrease in extracellular infectivity but no change in cell-associated infectivity. Nevertheless, extracellular virus still accounted for approx. 60% of the total infectious virus.

Effect of trypsin on EHDV infectivity

Results of the effect of trypsin on EHDV are given in Table 3. It can be seen that trypsin at 75 μg/ml inactivated EHDV as did trypsin at 5 μg/ml. The presence of 5 μg/ml trypsin in the culture medium similarly decreased the recovery of infectious virus.
Fig. 4. EHDV-infected cell monolayer 24 h post-infection. (a) Unstained cell observed with phase-contrast optics to illustrate the characteristic protuberances or ‘buds’ (arrows). (b) The same cell stained with fluorescent antibody showing the localization of virus antigen in the ‘buds’ (arrows).

**Light microscopic observations**

EHDV infection of BHK-21 cells led to rounding and shrinkage of cells and eventual detachment. Typical perinuclear virus inclusions first became visible at about 10 h post-infection. These inclusions were eosinophilic when stained with haematoxylin–eosin. They also displayed the staining characteristics of double-stranded nucleic acid when stained with acridine orange. Fluorescent antibody staining demonstrated that these inclusions contained virus antigens. Fig. 3 shows such inclusions in infected BHK cells. At approx. 20 h post-infection small- to large-sized localized swellings could be seen in infected cells. One to three swellings were normally present per cell, each with a diam. averaging 10 μm. These swellings appeared to ‘bud off’ from the cells. Staining by the haematoxylin–eosin, acridine orange and fluorescent antibody methods demonstrated that these ‘buds’ had the characteristics of virus inclusions. Fig. 4 (a, b) shows an EHDV-infected cell with ‘buds’. Fig. 4 (a) is a phase-contrast view of the cell and Fig. 4 (b) is the same cell stained by the fluorescent antibody technique demonstrating the presence of virus antigens in the ‘buds’.

**Electron microscopic observations**

Early in infection, virus matrices, tubules and often paracrystals could be seen to form in perinuclear locations in infected cells. A low magnification photograph (Fig. 5) shows several developing inclusions near which are many tubular forms. Tubules, with a diam. of approx. 50 nm, and paracrystals, with a lattice spacing of 9 to 11 nm, were the first virus-associated structures to be seen in the course of infection (Fig. 5, 6, 7, 8). They could normally be detected at approx. 6 h post-infection. Shortly thereafter, homogeneous virus matrices could be seen to form. By approx. 18 h post-infection virus particles had matured from the matrices (Fig. 8). At this time and thereafter, portions of the cell membrane were seen to break down and release virus particles and some cellular constituents (Fig. 9). Virus particles were not released from the cell individually but seemed to egress in aggregates. It is also important to
EHDV replication in BHK-21 cells

Fig. 5. Electron micrograph of BHK-21 cell 8 h after infection. The cytoplasm contains several virus inclusions consisting of granular inclusions surrounded by many tubular forms. n, Nucleus; m, mitochondria; vi, virus inclusions; t, tubules. Bar marker represents 1 μm.

note that infected cells did not disintegrate in a generalized manner but instead localized cell membrane breakdown occurred in proximity to virus aggregates (Fig. 10).

Examination of sonicated extracellular virus preparations revealed virus particles to be present singly or in groups of two or three. Large aggregates of up to 50 to 60 virus particles as seen in unsonicated preparations (Fig. 9) were not seen in sonicated preparations. Core particles were seen in the ratio of about 1 core particle to 100 intact virus particles in sonicated EHDV preparations.
Fig. 6. Higher magnification of infected cell showing granular virus inclusions (vi), tubules (t) and a few early virus particles (vp); er, endoplasmic reticulum. Bar marker represents 1 μm.

Fig. 7. Infected cell showing a granular virus inclusion (vi) and a crystalline form (c) adjacent to each other. Bar marker represents 1 μm.

DISCUSSION

Typical EHDV cytopathic effect as described previously (Tsai & Karstad, 1970; Bando, 1975; Kontor & Welch, 1976; Fosberg et al., 1977; Lawman et al., 1977) was observed in this study. Similarly, perinuclear immunofluorescence as reported by Jochim et al. (1974) and
cytoplasmic double-stranded type fluorescence using acridine orange staining as described by Fosberg et al. (1977) were also observed, although in the latter case we observed the fluorescence to be present in discrete inclusions. We also observed distinct eosinophilic perinuclear inclusions after haematoxylin–eosin staining. All these inclusions undoubtedly
represent areas of virus-associated material as the location of the inclusions corresponds with the location of virus matrices, tubules and virus particles as seen by electron microscopy. The features and structures associated with EHDV infection as seen by electron microscopy correspond to previous reports (Tsai & Karstad, 1970, 1973; Murphy et al., 1971; Thomas & Miller, 1971; Huismans & Els, 1979). As indicated by Tsai & Karstad (1970), virus maturation appears to occur within matrices, the matrices seemingly reorganizing into virus particles. Virus-associated tubules did not appear to be of cellular origin and indeed Huismans et al. (1979) and Huismans & Els (1979) have shown that these tubules are composed of a virus protein. The paracrystals observed in infected cells also seemed to be of virus origin for similar structures were not observed in uninfected cells. Furthermore, virus particles were frequently found associated with the paracrystals. As in the case with tubules the paracrystals may be composed of some virus component.

As infection proceeded, several novel features of EHDV replication were noted. Microscopically, inclusions seemed to migrate towards the cell periphery, frequently causing swelling of a portion of the cell and ultimate bursting of that area. These swellings were large, averaging about 10 μm in diam., and are therefore quite distinct from the budding areas described by Thomas & Miller (1971) which contained only single virus particles. Similarly, virus aggregates, often incompletely surrounded by cellular remnants could be seen extracellularly. Significant amounts of virus are present extracellularly, although accurate quantification of extracellular virus could not be carried out without first sonicating the supernatant. We believe that sonication serves to break up the released virus aggregates and thus increase the number of infectious virus particles but we could not rule out the possibility that sonication might have enhanced infectivity by removal of the outer capsid of EHDV as seen with rotaviruses (Spendlove & McClain, 1967, 1968; Spendlove et al., 1970). Rotavirus infectivity is increased by the presence of trypsin in the culture medium (Babiuk et al., 1977; Almeida et al., 1978; Graham & Estes, 1980), and are also like EHDV, found in extracellular
aggregates (Esparza et al., 1980). However, we found that trypsin inactivates EHDV as found by Svehag et al. (1966) for bluetongue virus. The large increases in infectivity following sonication monitored by electron microscopy showed the disappearance of aggregates and an increase in intact virus particles.

EHDV release has been described as occurring through cell lysis (Tsai & Karstad, 1970; Murphy et al., 1971). Our observations showed that cell lysis occurred at sites near aggregations of virus particles as they moved towards the cell periphery and could be observed by light microscopy as swellings in the cells. Although an extensive search was made with the electron microscope for the large 'buds' seen by light microscopy, no good examples were found. This finding, or lack of, is not necessarily contradictory as infected cells were quite disrupted when observed by electron microscopy after 18 h post-infection whereas infected cells did not appear severely disrupted when examined up to 24 h post-infection by light microscopy. Examination of cell cultures at 20 h post-infection by electron microscopy showed that virus aggregates were often associated with cell constituents so that these associations might be remnants of the buds seen by light microscopy. The process of EHDV replication and release therefore showed a definite temporal pattern beginning at about 6 h with the formation of the tubules and paracrystals in the perinuclear region followed by maturation of virus particles. Infectivity studies (Tables 1 and 2) show that most infectious virus was extracellular although sonication was necessary to disrupt the virus aggregates to obtain maximum infectivity. In contrast is the study of Lawman et al. (1977) who found that most virus was cell-associated until 72 h when the supernatant virus approached the total virus. Since they did not disrupt the culture supernatant, their reported amounts of extracellular virus may not have accurately reflected the actual amount of infectious virus present. The time difference between the one-step growth curve presented in this study and that of Lawman et al. (1977) is not considered significant as EHDV replication proceeded at slower rates during earlier passages.

The replication time of EHDV decreased with serial passage. Similarly, virus yield increased albeit to a plateau. This phenomenon was observed for all EHDV strains and presumably represented an adaptation period of EHDV to our BHK-21 cells and culture conditions. However, differences in virus yields were seen between EHDV strains, probably because the degree of adaptation of the virus strains to BHK-21 cell culture varied. EHDV-ATCC and EHDV-Wis produced the greatest yields and were passaged extensively in cells prior to our acquisition of them. Similarly, low-yield strains such as EHDV-NJ and EHDV-ND did not have extensive cell passage histories. There was no correlation between yields and EHDV serotype. The proportions of supernatant and cell-associated virus remained quite constant between EHDV strains.

The research project was supported by funds from The Natural Sciences and Engineering Research Council of Canada.

REFERENCES


(Received 29 October 1981)