The ultrastructure of the developing replication site in foot-and-mouth disease virus-infected BHK-38 cells

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Foot-and-mouth disease virus (FMDV) is the type species of the Aphthovirus genus of the Picornaviridae. Infection by picornaviruses results in a major rearrangement of the host cell membranes to create vesicular structures where virus genome replication takes place. In this report, using fluorescence and electron microscopy, membrane rearrangements in the cytoplasm of FMDV-infected BHK-38 cells are documented. At 1–2 h post-infection, free ribosomes, fragmented rough endoplasmic reticulum, Golgi and smooth membrane-bound vesicles accumulated on one side of the nucleus. Newly synthesized viral RNA was localized to this region of the cell. The changes seen in FMDV-infected cells distinguish this virus from other members of the Picornaviridae, such as poliovirus. Firstly, the collapse of cellular organelles to one side of the cell has not previously been observed for other picornaviruses. Secondly, the membrane vesicles, induced by FMDV, appear distinct from those induced by other picornaviruses such as poliovirus and echovirus 11 since they are relatively few in number and do not aggregate into densely packed clusters. Additionally, the proportion of vesicles with double membranes is considerably lower in FMDV-infected cells. These differences did not result from the use of BHK-38 cells in this study, as infection of these cells by another picornavirus, bovine enterovirus (a close relative of poliovirus), resulted in morphological changes similar to those reported for poliovirus-infected cells. With conventional fixation, FMDV particles were not seen; however, following high-pressure freezing and freeze-substitution, many clusters of virus-like particles were seen.

INTRODUCTION

Foot-and-mouth disease virus (FMDV) is one of the most infectious pathogens known and is responsible for worldwide outbreaks of foot-and-mouth disease in cloven-hoofed animals. The 2001 outbreak in the United Kingdom devastated livestock farming and resulted in the slaughter of 4 million animals at an estimated cost of £10 billion.

FMDV is the type species of the genus Aphthovirus of the Picornaviridae (Belsham, 1993). Currently, eight other picornavirus genera are recognized including the cardioviruses [e.g. Encephalomyocarditis virus (EMCV)], entero-viruses [e.g. Poliovirus (PV), Bovine enterovirus (BEV) and echovirus 11 (EV11)], parechoviruses [e.g. parechovirus-1 (HpeV-1)] and rhinoviruses. Picornaviruses are small, single-stranded, positive-sense RNA viruses which have a non-enveloped icosahedral capsid (of approximately 25 nm diameter) formed from 60 copies each of four virally encoded proteins, VP1–VP4.

Members of the Picornaviridae have a similar genome organization and are believed to follow a similar replication strategy (King et al., 2000). Infection is initiated by virus binding to a specific cell surface receptor and culminates in the translocation of the viral RNA (vRNA) across a cellular membrane into the cytosol (Belnap et al., 2000; Miller et al., 2001; Schober et al., 1998). Inside the cytosol, the vRNA functions sequentially as a template for the synthesis of the viral proteins and the complementary negative-stranded genome copies that are, in turn, used as a template for the synthesis of new progeny genomes.

Infection by picornaviruses results in a major rearrangement of the host cell membranes to create vesicular structures upon which virus genome replication takes place (Bienz et al., 1983, 1987; Egger et al., 2002; Frankel et al., 1987; Gosert et al., 2000, 2002). The precise origin of these vesicles is not clear and, for PV, a number of cytoplasmic organelles have been implicated in their derivation. At early
times following infection, PV-induced vesicles are most likely formed at the endoplasmic reticulum (ER) (Dales et al., 1965; Rust et al., 2001; Schlegel et al., 1996; Suhy et al., 2000) whereas, at later times, membranes from other cellular organelles, including lysosomes and the Golgi, may also contribute to vesicle formation (Rust et al., 2001; Schlegel et al., 1996).

Despite having a similar replication strategy, several studies have revealed important differences in the replication of picornaviruses from different genera. These studies have suggested that virus genome replication may take place on vesicles derived from different cellular membranes and at a different site within the cell (Gazina et al., 2002; Rust et al., 2001). The membrane vesicles induced, following infection by PV, EV11 and EMCV, appear to have a similar morphology consisting of heterogeneously sized vesicles arranged as tightly packed clusters. However, the vesicles induced by HpeV-1 (Gazina et al., 2002) are distinct from the other picornaviruses studied in that they are homogeneously sized, less numerous and do not associate to form tight clusters. Another important difference in picornavirus replication is revealed by their sensitivity to brefeldin A (BfA). BfA inhibits membrane transport between the ER and the Golgi by preventing the formation of COPI-A (BfA). BfA inhibits membrane transport between the ER and the Golgi by preventing the formation of COPI-A (BfA). BfA inhibits membrane transport between the ER and the Golgi by preventing the formation of COPI-A (BfA). BfA inhibits membrane transport between the ER and the Golgi by preventing the formation of COPI-A (BfA). BfA inhibits membrane transport between the ER and the Golgi by preventing the formation of COPI-A (BfA).

Whilst there is considerable information on the morphological changes that take place within picornavirus-infected cells (Bienz et al., 1987, 1994; Dales et al., 1965), little is known about the nature of any membrane rearrangement and the development of a specialized area of virus replication in FMDV-infected cells. Wool et al. (1982) and Yilma et al. (1978) reported FMDV in cytoplasmic blebs, and, more recently, cytoplasmic blebs were reported to be a feature of BHK-21 cells persistently infected with FMDV, although the authors were unable to correlate these structures with the presence of virions (Donn et al., 1995).

In the absence of a definitive study of FMDV-infected cells, we have performed an ultrastructural study of BHK-38 cells infected with FMDV. This study has shown that FMDV infection results in a dramatic condensation of the cytoplasmic contents to a distinct region, located to one side of the cell nucleus, which contains both viral proteins and RNA. The cytoplasm between this region and the plasma membrane appears generally devoid of recognizable cellular structures apart from a ring of mitochondria which surrounds the accumulated organelles. Prominent within this region are densely packed free ribosomes and smooth membranous vesicles that have no counterpart in the uninfected cell. These vesicles are distinct from those induced by enteroviruses and more closely resemble those induced by HpeV1, since they are relatively few in number and do not aggregate into densely packed clusters (Gazina et al., 2002). It could be argued that the membrane rearrangements induced by FMDV were characteristic of the cell type (BHK-38) and not the virus (FMDV). However, when the same cell type was infected with BEV the resultant series of morphological changes did not resemble those seen with FMDV but were similar to those reported for cells infected with PV.

**METHODS**

**Cell culture, viruses and reagents.** BHK-38 cells were obtained from ECACC, cultured in DMEM supplemented with 10% fetal calf serum (FCS), 20 mM glutamine, penicillin (100 U Units ml$^{-1}$) and streptomycin (100 μg ml$^{-1}$) at 37 °C in 5% CO₂. Stocks of FMDV O,BFS and BEV were prepared and virus titres determined using BHK-38 cells as described previously (Jackson et al., 1996) except that, for BEV, horse serum was used in place of FCS. In all subsequent experiments involving BEV, pig serum was used in place of FCS. The m.o.i. was based on the virus titre on BHK-38 cells. BfA was purchased from Sigma and a stock solution (5 mg ml$^{-1}$) prepared in DMSO.

**Cell infections**

**One-step growth curves.** Virus (m.o.i. = 5 p.f.u. per cell) was adsorbed to BHK-38 monolayers in 60 mm dishes at room temperature for 0.5 h in the presence of 5 μg BfA ml$^{-1}$ or DMSO (1 in 1000; mock-treated). The cells were washed with PBS pH 7.5 and incubated at 37 °C in the presence of BfA (5 μg ml$^{-1}$) or DMSO in cell culture media supplemented with 1% FCS or pig serum for FMDV and BEV respectively. At various times, the cell culture medium was assayed for the presence of virus by plaque assay on BHK-38 cells as described previously (Jackson et al., 1996) except that for BEV pig serum was used in place of FCS.

**Microscopy.** Cells were seeded onto 13 mm glass coverslips (Agar Scientific) for immunofluorescence and onto 13 mm thermanox coverslips (Science Services) for electron microscopy 16 h before use. Cells prepared on coverslips as above, were washed with cold PBS containing 1 mM CaCl$_2$ and 1 mM MgCl$_2$ and incubated with virus at a m.o.i. of between 10–100 p.f.u. per cell for 0.5 h at 4 °C. The cells were washed with PBS to remove unattached virus and infection initiated by adding cell culture medium at 37 °C. Infected cells were then cultured at 37 °C.

**Fluorescence immunolabelling.** Coverslips of infected and uninfected cells (control) were fixed at 1.5, 2.0, 2.5 and 3.0 h post-infection (h p.i.) for 0.5 h in 4% paraformaldehyde in PBS, washed in PBS and stored at 4 °C. For immunolabelling, cells were permeabilized at 0–0.5 % Triton X-100 in PBS for 15 min and washed in PBS. Non-specific binding of antibody was blocked by 0.5 h incubation in PBS containing 0.5% bovine serum albumin (Sigma) (PBS/BSA). Cells were immunolabelled by incubation in rabbit antiserum raised against whole FMDV diluted 1:600 in PBS/BSA or mouse anti-tubulin antibody (Sigma) at 1:1000 for 1 h at room temperature followed by PBS washes (3 × 5 min). Primary antibody was detected with Alexa 488- or Alexa 568-conjugated species-specific immunoglobulins (Molecular Probes) diluted 1:200 in PBS/BSA. Incubation was for 1 h at room temperature followed by PBS washes (3 × 3 min). Nuclei were labelled with ToPro 3 (Molecular Probes) diluted 1:5000 in PBS for 5 min. Coverslips were mounted in Vectashield (Vector Laboratories), sealed with nail varnish and were imaged in a Leica TCS SP2 confocal microscope. Control uninfected cells were labelled as for infected cells.
RNA labelling and localization. Newly synthesized FMDV RNA was detected by bromouridine (BrU) incorporation. Cells on coverslips were fixed with FMDV and then transfected with Bromo-UTP (BrUTP) using Lipofectin (Gibco-BRL) according to the manufacturer’s instructions. Briefly, 10 μl of Lipofectin were added to 90 μl of OptiMEM (Gibco-BRL). After 10 min at room temperature, 100 μl of 10 mM BrUTP, diluted in OptiMEM, was added and the mixture incubated at room temperature for a further 45 min. The mixture was further diluted with 1 ml OptiMEM that contained 20 μg actinomycin D and added to cells immediately following FMDV infection. Control cells were processed in parallel. Control cells were transfected either (1) in the absence of infection; (2) in the absence of actinomycin D or (3) in the absence of BrUTP in the transfectant medium. Transfected cells were incubated at 37°C in 5% CO₂. At 2 h p.i., the cells were fixed for 1 h in 4% paraformaldehyde in PBS and washed in PBS. Cells were permeabilized in 0.05% Triton X-100 in PBS for 15 min. FMDV proteins were identified using a rabbit anti-FMDV antiserum (as above) and newly synthesized viral RNA was detected using a mouse anti-BrdU (Boehringer Mannheim Biochemica), diluted 1:10 and incubated as described by the manufacturer. Primary antibodies were detected with anti-mouse Alexa 568- and anti-rabbit Alexa 488-conjugated species-specific immunoglobulins (Molecular Probes) diluted 1:200 in PBS/BSA. The nuclei were labelled with 4',6-diamidino-2-phenylindole (DAPI, Molecular Probes).

Routine electron microscopy of adherent cells. At 1-5, 2-0, 2-5 and 3-0 h.p.i. control uninfected and FMDV-infected cells on Thermanox coverslips were fixed in situ in 2% glutaraldehyde in 0.05 M phosphate buffer (pH 7.2–7.4, osmotic pressure adjusted to 350 mosmol with sucrose) for a minimum of 2 h. BHK-38 cells infected with BEV were fixed at 2-0, 4-0, 6-0 and 8-0 h p.i. and processed as for FMDV-infected cells. Samples were post-fixed in 1% osmium tetroxide in phosphate buffer for a minimum of 4 h, dehydrated through a graded series of ethanol and embedded in Agar 100 resin. After overnight polymerization at 60°C the thermanox coverslips were peeled from the resin block leaving the cells embedded in the resin. Sections were cut parallel to the coverslip surface on a Diatome diamond knife (Leica Microsystems) and contrasted with uranyl acetate and lead citrate with an EMStain coverslip surface on a Diatome diamond knife (Leica Microsystems). Sections were cut parallel to the thermanox coverslips were peeled from the resin block leaving the cells embedded in the resin. Routine electron microscopy of non-adherent cells. For cells fixed at 2-0, 2-5 and 3-0 h.p.i., the tissue culture medium was removed prior to fixation and centrifuged at 1000 g for 5 min. The medium was removed and the cell pellet consisting of infected cells that had rounded up and detached from the coverslip was fixed in 2% glutaraldehyde, washed in phosphate buffer and embedded as a pellet in 8% gelatin. Subsequent processing was as for adherent cells.

High-pressure freezing and freeze-substitution. Twenty-five cm² flasks of BHK-38 cells were harvested at 2-0, 2-5 and 3 h p.i. Flasks were firmly shaken and a detached cells were collected by removal of the medium and centrifugation at 1000 g for 5 min. The supernatant was removed and the cells mixed with a small residual quantity of medium to form a thick suspension. This was introduced into 200 μm diameter cellulose tubing (Hohenberg et al., 1994) placed in hexadecene within 200 μm planchettes and frozen in a Bal-Tec HPM 010 high-pressure freezer (Bal-Tec, Liechtenstein). After freezing, the planchettes were separated under liquid nitrogen and the planchettes containing the cellulose tubes were transferred to a Leica AFS for freeze-substitution. This was done essentially as described previously (Monaghan et al., 1998). Samples were freeze-substituted in acetone containing 1% osmium tetroxide. Freeze-substitution was undertaken at −90°C for 48 h, −60°C for 8 h, and −30°C for 8 h. The samples were then warmed to 0°C and the acetone/osmium tetroxide replaced with fresh acetone. Finally the samples were warmed to room temperature and embedded in Agar 100 resin.

RESULTS

Brefeldin A experiments

Picornaviruses display a differential sensitivity to BfA. FMDV has been shown to be resistant to BfA by O’Donnell et al. (2001). In that study BfA was added to the cells at 1 h.p.i. Since infection of BHK-38 cells with the FMDV strain used in our study results in extensive CPE within 2–3 h we added BfA at the same time as the virus. Fig. 1 shows that, under these conditions, the replication of FMDV is insensitive to BfA whereas the replication of

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**Fig. 1.** Effect of brefeldin A on replication of FMDV and BEV. (a) FMDV O₁B₁S₁ or (b) BEV were adsorbed onto BHK-38 monolayers (m.o.i.=5 p.f.u. per cell) for 30 min at room temperature in the presence or absence of 5 μg brefeldin A (BfA) ml⁻¹. The cells were then washed and incubated for increasing periods of time at 37°C again in the presence or absence of 5 μg BfA ml⁻¹. At the times indicated, the cell culture medium was assayed for the presence of virus by plaque assay on BHK-38 cells.
BEV is significantly inhibited. To confirm that BfA does not inhibit replication of FMDV we also treated cells with BfA for 0–5 h prior to, and during, infection. Under these more vigorous conditions replication of FMDV was still not inhibited by BfA (data not shown).

**Immunofluorescence microscopy**

To establish the kinetics of infection, FMDV-infected BHK-38 cells were monitored for the presence of virus proteins over time by immunofluorescence microscopy (Fig. 2). The effects of FMDV on BHK-38 cells were rapid with visible signs of cytopathic effect apparent at 2.5 h p.i. The infected cells changed from a flattened morphology to a more rounded shape and, by 3 h p.i., the majority of the cells were floating in the culture medium having become detached from the substrate.

In Fig. 2(a), BHK-38 cells infected with FMDV were labelled with antibodies specific to α-tubulin in order to demonstrate the cytopathic effect. The two cells at the bottom of the image show a flattened morphology and extended cytoskeleton characteristic of uninfected cells. The cells at the top of the image show the typical cytopathic effect produced by FMDV where the cells have rounded up. When monitored by immunofluorescence, many of the cells in infected cultures demonstrated detectable FMDV labelling in a discrete region on one side of the nucleus within 1.5–2.0 h p.i. (Fig. 2b). As the time post-infection increased, this labelling expanded to fill virtually all of the cytoplasm (Fig. 2c, d).

**Localization of RNA**

We followed incorporation of BrUTP into newly synthesized viral RNA molecules using an anti-BrdU antibody, which is cross-reactive for BrUTP. BHK-38 cells were infected with FMDV and transfected with BrUTP in the presence of actinomycin D. Approximately 5–10% of the infected cells were positive for BrUTP staining. Fig. 3 shows a number of FMDV-infected cells, one of which is showing positive labelling for BrUTP incorporation. In these double-labelled cells, the subcellular patterns of labelling for the FMDV protein (Fig. 3a) and BrUTP-labelled viral RNA (Fig. 3b) were similar (Fig. 3c). The antiserum used in these studies predominantly recognizes structural proteins, but similar results have been obtained with antibodies detecting non-structural proteins (data not shown). Thus the region of the cell containing both viral proteins and virus-specific RNA is likely to be the site of virus replication and will be referred to as the replication site.

Control cells transfected without BrUTP in the transfection medium, or transfected but uninfected, were unlabelled with the anti-BrdU antibody. Cells transfected and infected in the absence of actinomycin D, labelled with the anti-BrdU antibody within the replication site and also showed some labelling within the nucleus.

**Electron microscopy of FMDV-infected BHK-38 cells**

Next we performed a detailed analysis of FMDV-infected BHK-38 cells by electron microscopy. The methods used to fix, embed and section the infected cells *in situ* allowed the changes taking place during virus infection to be studied without the risk of mechanically induced artefacts. Uninfected BHK-38 cells exhibit a standard mammalian cell complement of organelles with, for example, abundant Golgi, rough ER (rER), free ribosomes and mitochondria (see Fig. 5b).

The gross changes in cell structure observed by confocal microscopy (Fig. 2) were seen in greater detail by electron microscopy, and many of the morphological changes in the BHK-38 cells correlate with the time-course of infection. However, the precise chronology of the structural consequences of FMDV infection was difficult to determine, as cultures fixed at different times contained cells showing a range of effects. Nonetheless, at each time point examined, the majority of infected cells showed similar altered features. Thus, the results have been interpreted in terms of a basic time course indicated by three successive stages; early, mid and late.

**Early phase: 1.5–2.0 h p.i.** The first morphological indication of virus infection that can be unequivocally identified by electron microscopy is the accumulation of cytoplasmic organelles in a region to one side of the nucleus (Fig. 4). This region has the same location within the cell as the early replication site identified by immunofluorescence (Figs 2c, 3c).

The developing replication site is characterized by the accumulation of rER and Golgi. The rER showed a subtle change in that the membranes appeared less distinct and the numbers of ribosomes on the membranes were reduced when compared with uninfected cells. Conversely, the number of ribosomes free within the replication sites increased which may indicate the loss of ER-associated ribosomes as a possible source. The mitochondria were largely restricted to a peripheral region of the developing replication site (Fig. 4a, b), although occasional mitochondria were also seen in the rest of the cell cytoplasm that was otherwise relatively sparsely occupied by organelles.

At this stage in infection, Golgi stacks with normal appearance and a small number of smooth, membrane bound vesicles were seen (Fig. 4c). These structures have no counterpart in normal cells.

**Mid-phase: 2.0–2.5 h p.i.** The majority of cells in cultures 2.0–2.5 h p.i. had a well-developed replication site. The rER was almost devoid of ribosomes and had fragmented considerably, although larger fragments remained at the outer region of the replication site (Fig. 5a). In addition, the nuclear membrane showed blebbing similar to that seen in the cytoplasmic rER (supplementary images: www.iah.bbsrc.ac.uk/bioimaging/jgv). In comparison,
Fig. 2. Cytopathic effect caused by FMDV observed by fluorescence microscopy. (a) BHK-38 cells were infected with FMDV O1 BFS for 3 h. Cells were fixed, permeabilized and incubated with antibodies specific for α-tubulin and appropriate secondary antibodies coupled to Alexa 488 to locate the microtubule network (green). Nuclear DNA was labelled with ToPro 3 (blue). The cells in the lower part of the image show the flattened morphology characteristic of uninfected BHK cells. The cells in the upper part of the image show rounded morphology characteristic of the cytopathic effect. Bar, 6 μm. (b), (c) and (d) BHK-38 cells were infected with FMDV and fixed at increasing times post-infection. Cells were permeabilized and viral capsid proteins were located using a rabbit polyclonal antibody specific for the viral capsid proteins followed by secondary antibody coupled to Alexa 488 (green). Nuclear DNA was labelled with ToPro 3 (blue). Cells were imaged by transmitted light in order to define the outline of the cell and intracellular organelles (grey scale). (b) An early stage post-infection where low levels of FMDV capsid protein are present next to the nucleus. (c) A mid stage in infection where FMDV capsid protein is seen to accumulate to one side of the nucleus. (d) Late stage of infection where cells have rounded and the FMDV protein fills the cytoplasm. Bar, 2 μm.
Fig. 5(b) shows the region of cytoplasm adjacent to the nucleus in an uninfected cell. The cytoplasm contains rER with attached ribosomes and intact nuclear membrane.

Within the replication site, the number of membrane-bound vesicles increased and they were often seen in close proximity to the remnants of the rER (Fig. 6a). Whilst in the early replication site these vesicles were almost always lined by a single membrane, from 2·0 h p.i. a small number were lined by a double membrane (Fig. 6b). These double membrane vesicles formed less than 10% of the total vesicle population.

The apparent electron density of the replication site appeared to result from the accumulation of single and small clusters of ribosomes. However at this mid-phase of replication, clusters of ribosomes within the replication site were organized in straight lines (Fig. 6c). This ribosomal ordering is not seen in uninfected cells or at later times post-infection. At this mid-replication phase, the Golgi regions were less prominent, appeared less well organized, and were dispersed throughout the replication site.

Late phase: 2·5–3·0 h p.i. At this stage of infection the majority of infected cells were rounded (Fig. 2d) and the cytoplasm was composed, almost exclusively, of the replication site (Fig. 7). Small remnants of the rER were scattered throughout the replication site. The ribosomes were no longer arranged in the ordered chains seen in the mid-phase of infection but were randomly dispersed throughout the replication site. Membrane vesicles were prominent and remain a feature of cells recovered from the medium at all later times post-infection (see below). As in the mid-phase of infection, the majority of these vesicles appeared to be bounded by a single membrane, although...
Fig. 4. Ultrastructural changes in cells at early stages of infection with FMDV. BHK-38 cells were processed for transmission electron microscopy between 1.5 and 2.0 h p.i. with FMDV O1 BFS. (a) Low-magnification image. The majority of the cytoplasmic organelles including the Golgi and rough endoplasmic reticulum (rER) have accumulated in the perinuclear region while the rest of the cytoplasm is relatively devoid of organelles. Bar, 2 μm. (b) Medium power image where mitochondria are seen to line the edge of the area containing cellular organelles. Bar, 1 μm. (c) High-magnification image of the redistributed organelles. The Golgi has normal stacked appearance (short arrow) and the rER has fragmented and lost the majority of associated ribosomes. A small number of membranous vesicles are also present (long arrow). Bar, 300 nm.
Fig. 5. Ultrastructural changes in cells at mid-stages of infection with FMDV observed at medium power. BHK-38 cells were processed for transmission electron microscopy between 2 and 2.5 h p.i. with FMDV O1 BFS. (a) The replication site occupies a significant proportion of the cytoplasm. The rER is fragmented with larger remnants located at the periphery of the site. The rER is almost totally devoid of ribosomes and the Golgi stacks are dispersed. Mitochondria are evident on the edge of the site. A small number of membranous vesicles are present (arrow). (b) Shows an equivalent region of an uninfected cell. Golgi and rER membranes are indicated. Bars, 1000 nm.
Fig. 6. Ultrastructural changes in cells at mid-stages of infection with FMDV observed at high power. BHK-38 cells infected with FMDV O₁BFS were processed for transmission electron microscopy as described in the legend to Fig. 5. (a) Large numbers of vesicles are present (arrow) within the replication site. These contain electron-dense material and are often associated with membranes that have the appearance of ribosome-depleted rER. Bar, 100 nm. (b) Vesicles with double membranes are occasionally seen (arrow). (c) Within the replication site a large number of ribosomes are present in linear arrays. Bar, 200 nm.
occasional double membrane structures were seen. These vesicles were dispersed throughout the replication site and did not form ordered clusters.

**Electron microscopy of BEV-infected BHK-38 cells**

The morphological changes that take place within FMDV-infected cells were strikingly different from those reported following infection of cells by other picornaviruses. In particular, the dramatic collapse of the cytoplasmic contents to form a replication site has not been previously reported for cells infected by other picornaviruses. This difference could result from the use of different cells from those used in studies involving other picornaviruses. Therefore, to determine whether the changes that take place were unique to FMDV, we investigated the membrane rearrangements that occur following infection of BHK-38 cells with BEV, an enterovirus in the same genus as PV.

The time course of the infection was longer for BEV than for FMDV-infected cells (see Fig. 1); nonetheless it was possible to identify similar time points in the progress of infection. The cytoplasmic changes in BEV-infected cells were different from those induced by FMDV and resembled more closely the structures reported for cells infected by poliovirus. In the early phase of infection, the initial changes were seen in a similar location to FMDV-infected cells but the accumulation of virtually all cellular organelles within this region was not as pronounced. At the mid-phase of infection the rER was disordered throughout the cytoplasm and within the region of condensed organelles was extensively fragmented. In contrast to FMDV-infected cells, the ribosomes remained associated with membrane fragments of the ER.

From the mid-phase of infection, membrane vesicles, similar to those induced by PV, were seen within BEV-infected cells. They did not contain the fibrillar material seen in the FMDV-infected cells and the majority were bound by a single membrane. In addition, like PV, the vesicles were of variable size and were seen to accumulate into rosette-like formations.

At the later stages of infection, BEV did not cause the total collapse of the cell organelles round the replication site seen in FMDV-infected cells (supplementary images: www.iah.bbsrc.ac.uk/bioimaging/jgv). However, unlike in

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**Fig. 7.** Ultrastructural changes in cells at late stages of infection with FMDV. BHK-38 cells were processed for transmission electron microscopy between 2-5 and 3 h p.i. with FMDV O1 BFS. The cell is reduced in volume and the replication site fills most of the cytoplasm. Many membranous vesicles can be seen dispersed throughout the replication site. Bar, 1000 nm.
the FMDV-infected cells, virus-like structures were readily apparent. In the later stages of infection, these structures accumulated into paracrystalline arrays (Fig. 8c), but were also often seen in association with the rosettes of vesicles (Fig. 8d).

High-pressure freezing of FMDV-infected cells

Throughout these studies, no virus-like particles were observed in FMDV-infected cells processed for conventional electron microscopy. High-pressure freezing is the optimal

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**Fig. 8.** Comparison of ultrastructural changes in cells at late stages of infection with FMDV and BEV. FMDV: (a) and (b) BHK-38 cells were processed by high-pressure freezing and transmission electron microscopy 3 h p.i. with FMDV O1 BFS. (a) Low-power image indicates areas within the replication site that contain virus-like particles (arrows). Bar, 500 nm. (b) Higher power view which shows virus-like particles (arrow). Note, several virus-induced membrane vesicles have electron-dense cores. Bar, 200 nm. BEV: (c) and (d) BHK-38 cells were processed by conventional transmission electron microscopy 5 h p.i. with BEV. (c) A high-power image shows an accumulation of virus-like particles in the replication site. Bar, 200 nm. (d) A high-power image shows that the vesicles induced by BEV are heterogeneous in size and some are clustered in rosettes (arrow). They do not contain the electron-dense material seen in the FMDV-induced vesicles in Fig. 6(a). Small clusters of virus-like particles are also present (arrowhead). Bar, 100 nm.
method for preparing cells for electron microscopy in that it avoids artefacts of aldehyde fixation whilst giving the maximum volume of ice-artefact-free preservation. In addition, both physical and enzymic methods for cell removal may result in changes to cell architecture (P. Monaghan, unpublished observations). Therefore, we used this method to visualize infected cells. At 3 h p.i., floating cells were collected from the medium and subjected to high-pressure freezing. Many of the infected cells collected in this way had not undergone cell lysis and remained intact during processing for the EM.

Several membrane-bound vesicles were present in infected cells and these were characterized by contents with variable electron density. These vesicles were similar to those seen in the routine electron micrographs in that they did not form clusters, and the majority appeared to have only a single membrane. In addition, many of the cells contain clusters of particles with an approximate diameter of 25 nm which could correspond to FMD virions (Fig. 8a, b and see Discussion).

**DISCUSSION**

Infection by RNA viruses results in a major rearrangement of the host cell membranes to create vesicular structures upon which viral RNA replication takes place (Bienz et al., 1987; Egger et al., 2002; Gosert et al., 2002). In this report we describe sites of membrane rearrangements in FMDV-infected BHK-38 cells and we also demonstrate that this feature of FMDV-infected cells is the site of viral RNA synthesis.

It is clear from the results presented that the morphological changes that take place within FMDV-infected cells are strikingly different from those reported following infection by other picornaviruses. For example, the earliest ultrastructural change that can be reliably recognized in cells infected with FMDV (at around 1·5–2·0 h p.i.) is a dramatic accumulation of virtually all of the cytoplasmic organelles to a defined region of the cell, which we describe as the virus replication site. This difference was unlikely to be BHK-38 specific since the morphological changes that take place in these cells infected with BEV, a close relative of PV, were similar to those reported for PV in Hep-2 cells (Bienz et al., 1994) and EV11 in BS-C-1 cells (Gazina et al., 2002).

The replication site became the main focus of the subsequent events that take place within the FMDV-infected cell. As this site developed, the rER became distorted and eventually fragmented into small remnants although larger areas of rER remained at the periphery of the replication site. Alterations were also seen in the nuclear membrane which was dilated and extended into the cytoplasm. The number of ribosomes associated with the rER was greatly reduced. At the same time, free ribosomes accumulated in the replication site. The loss of ribosomes from the rER membranes might indicate that at least some of these free ribosomes are derived from rER. At the mid-phase of infection, ribosomes were seen to associate into long straight chains. The significance of the appearance of chains of ribosomes is unclear. Certainly, this is a stage of rapid protein synthesis and it is possible that these structures represent actively translating viral RNA molecules. Taken together, these observations point to a major modification of the rER in the development of the replication site, notwithstanding the possible role for the rER in the genesis of the membrane-bound vesicles (see Introduction). However, rER redistribution was not noted as a feature of FMDV-infected cells when studied by immunofluorescence microscopy (O’Donnell et al., 2001), but this discrepancy may be explained by the increased resolution of the electron microscope.

The changes in the rER appeared to occur before any structural changes in the Golgi region, despite the fact that the first signs of de-novo FMDV protein synthesis was detected in the Golgi-rich area of the cells (data not shown). However, a later feature of the replication site is the loss of normal-appearing Golgi structures, and this is consistent with reports of the loss of Golgi organization in PV (Sandoval & Carrasco, 1997) and FMDV-infected cells (O’Donnell et al., 2001).

As infection progressed, membrane-bound vesicles became more numerous within the replication site and were a prominent feature of cells in the late stage of infection. These vesicles appear distinct from those induced by enteroviruses such as poliovirus and EV11 and more closely resemble those induced by HpeV-1, since they were relatively few in number and did not appear to aggregate into the rosettes or clusters (Bienz et al., 1994; Gazina et al., 2002). A second major difference is that the proportion of vesicles with double membranes is considerably less in FMDV-infected cells than in cells infected with PV. Again these differences are not explained by the use of BHK-38 cells, since the vesicles induced by BEV were similar in appearance to those seen in PV-infected cells. From their morphological characteristics it was not possible to determine the origins of the vesicles induced by FMDV but there was a close association between areas rich in these vesicles and the remnants of the rER, as illustrated in Fig. 6a. This association of virus-induced vesicles and fragments of the ER is consistent with previous observations made with PV (Bienz et al., 1987).

The precise origins of the membrane vesicles induced by picornavirus infection has been the subject of some debate although it is generally considered that, at least early in infection, they are derived from vesicles trafficking between the ER and the Golgi. Rust and colleagues (Rust et al., 2001) have shown that vesicles induced following infection by PV are formed at the ER by a budding mechanism that is similar to COPII-dependent formation of anterograde transport vesicles. Recently, Gazina et al. (2002) have observed components of COPI-dependent anterograde
transport vesicles associated with EV11, but not with EMCV-induced vesicles, leading the authors to suggest that the differential sensitivity of picornaviruses to BfA (see Introduction) may be explained if the vesicles they induce are formed at BfA-sensitive or BfA-insensitive steps of the anterograde transport pathway. Based on these observations and the insensitivity of FMDV to BfA (Fig. 1), it would be predicted that the vesicles induced by FMDV would be formed by a similar mechanism to that used by EMCV. However, further work will be required to determine if these vesicles retain a similar set of markers from the secretory pathway as described for other picornaviruses.

The morphology of the FMDV-induced vesicles appeared different depending upon the preparation method, although these vesicles are clearly the same structures. In conventionally fixed samples, vesicles contained a fine lattice-work of electron density, whereas in samples prepared by high-pressure freezing they have a dense core. The latter appearance corresponds to the vesicles seen in PV-infected cells (Schlegel et al., 1996) also prepared by high-pressure freezing; however, the prominent double membrane of these vesicles was not commonly seen in the FMDV-infected cells. When double membrane structures were detected in the FMDV-infected cells, it was almost exclusively in the mid to late stages of infection.

The issue of when the virus particles begin to form in the infected cell remains unresolved. Certainly, they must be present at later stages of infection. The failure to observe newly formed FMDV particles within any of the conventionally fixed cells is difficult to explain, given that virus-like particles were seen in BEV-infected cells prepared in an identical manner. However, FMDV is acid-labile, and when exposed to pH values below neutrality will dissociate. Although buffered, it may be that conventional fixatives for electron microscopy allow virions to be exposed to a brief period of low pH during the fixation process. This may explain the paucity of convincing electron micrographs of FMDV in infected cells using conventional processing. This explanation, that the virions are degraded during sample preparation, is supported by the presence of virus-like structures in samples prepared using high-pressure freezing and freeze-substitution.

In conclusion, we have shown that, like for other picornaviruses, infection of host cells by FMDV results in a major rearrangement of intracellular membranes. However, many of the cellular structural events resulting from FMDV infection differ markedly from the responses reported for other viruses within the Picornaviridae.

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REFERENCES


