Early Events in the Interaction Between Foot-and-Mouth Disease Virus and Primary Pig Kidney Cells

By D. CAVANAGH, D. J. ROWLANDS AND F. BROWN

Animal Virus Research Institute, Pirbright, Surrey, U.K.

(Accepted 23 May 1978)

SUMMARY

Foot-and-mouth disease virus (FMDV) attached to pig kidney cells at 0 °C and could only be recovered in a form with a sedimentation coefficient and buoyant density lower than that of the native virus. Incubation of the virus-cell complex at 37 °C caused disruption of about 80% of the particles into a 12S protein sub-unit that had the same polypeptide composition as that produced by reducing the pH of the virus below pH 7. The remaining 20% had the same polypeptide and RNA composition as the native virus but it had a lower sedimentation coefficient, buoyant density and specific infectivity. These lower values are probably due to the association of the virus with cell membrane components. The 12S subunits were shown to be located inside the cell, indicating that disruption of the virus had occurred within the cell. The results are discussed in relation to the different cell mediated alteration of other picornaviruses.

INTRODUCTION

The fate of picornaviruses after they have attached to host cells has been studied with poliovirus (Joklik & Darnell, 1961; Fenwick & Cooper, 1962; Holland, 1962; Mandel, 1962a, b; Lonberg-Holm et al. 1975; De Sena & Mandel, 1976, 1977), Coxsackie B3 virus (Crowell et al. 1971), the cardioviruses (Hall & Rueckert, 1971) and rhinoviruses (Lonberg-Holm & Korant, 1972; Lonberg-Holm & Noble-Harvey, 1973; Noble & Lonberg-Holm, 1973). Although previous studies of the early interaction of FMDV with pig kidney cells were made before purified radioactive virus was available (Brown et al. 1961, 1962; Thorne, 1962) so that a precise study of the early events was difficult, it was ascertained that the 140S virus particles were rapidly broken down to the virus RNA and a 12S sub-unit. In the present work purified radioactive virus has been used to study in more detail the fate of the virus capsid during interaction with pig kidney cells.

METHODS

Virus growth and assay. Three serotypes of FMDV were used; type O, strain 1; type A, strain 61; and type C, strain 997. Each virus was grown in BHK 21 cell monolayers and radioactively labelled with 35S-methionine or 3H-amino acids as previously described (Sangar et al. 1976). Virus infectivity was titrated by the plaque method on BHK 21 cell monolayers.

Purification of virus and 12S sub-units. Virus particles were prepared as described by Sangar et al. (1976). Virus 12S sub-units were prepared by disrupting virus particles at pH 6.5 with NaH2PO4 and purified by sedimentation at 95,000 g for 16 h in 5 to 25% linear sucrose gradients in 0.1 M-tris, 0.1 M-NaCl, pH 7.6.
**Pig kidney cells.** Primary pig kidney (PK) cells were grown in Roux bottles as described by Sellers (1955). The growth medium was Earle's saline supplemented with 0.01% yeast extract, 0.5% lactalbumin hydrolysate, 10% (v/v) bovine serum and antibiotics. The cells were removed from the glass with calcium-magnesium free phosphate buffered saline containing 0.01% EDTA and 0.01% trypsin. Bovine serum was then added to 10% (v/v), the cells sedimented, resuspended in phosphate-buffered saline (PBS), pH 7.4, passed through steel or nylon gauze and washed twice in PBS. The proportion of dead cells was estimated by staining with trypan blue.

**Interaction of virus with PK cells.** Radioactively labelled virus which had been purified in sucrose gradients was passed through a short column of Sephadex G100 in PBS containing 1 mg/ml bovine plasma albumin. The virus and PK cells in suspension were cooled in melting ice and the cells were then resuspended in the virus preparation for 15 to 30 min at 0 °C. The cells were sedimented and the extent of virus attachment was estimated by measurement of the radioactivity remaining in the supernatant. The cells were washed twice with cold PBS and resuspended in PBS pre-incubated to the desired temperature and the incubation continued in a water-bath. At intervals samples were removed, centrifuged at low speed and the supernatants estimated for eluted virus. The cells were washed twice with cold PBS, resuspended in 1 to 2 ml of PBS and usually sonicated for 15 to 30 s using an MSE 150 W ultrasonic disintegrator set at low power, amplitude 10. An equal vol. of either 2% (v/v) Nonidet NP40 or 2% SDS in water was then added to the disrupted cells. After 10 min at room temperature, the samples were centrifuged at 2000 g to sediment large undissociated material.

Cell extracts were then sedimented at 95000 g for 2 h in 18 ml 15 to 45% sucrose gradients in 0.04 M-phosphate buffer, pH 7.6. The sucrose contained 0.1% SDS or 0.1% NP40 corresponding to the detergent present in the extract. Fractions of 0.5 or 1.0 ml were collected and either 50 μl of each fraction were dried on to glass fibre discs or the entire fractions were mixed with an equal vol. of 20% trichloroacetic acid and the precipitate collected on glass fibre discs. The radioactivity on the discs was determined in a liquid scintillation counter.

**Buoyant density in caesium chloride.** The method used was described in detail by Rowlands et al. (1971). A modification of this procedure was the incorporation of a 2 ml pad of 22% sucrose in 0.04 M-phosphate buffer between the caesium chloride gradient and the sample. The pad prevented SDS in the sample from mixing with the caesium chloride, when precipitation would have occurred. The 5 ml pre-formed linear gradients, density 1.2 to 1.6 g/ml, were centrifuged at 70000 g for 6 h at 20 °C.

**Polyacrylamide gel electrophoresis.** Samples were electrophoresed on 8.5% polyacrylamide gel slabs using the continuous SDS-phosphate buffer system (Sangar et al. 1976). The gels were prepared for fluorography as described by Bonner & Laskey (1974) and exposed to preflashed Fuji film at −70 °C.

**RESULTS**

**Irreversible alteration of FMDV by pig kidney cells**

In most of the experiments to be described, about 10⁶ p.f.u. of 35S-methionine-labelled virus type 0-1 (equivalent to about 10¹¹ particles) were allowed to attach to 10⁸ suspended PK cells at 0 °C. About 80% (70 to 95% in different experiments) of the virus attached to the cells within 15 min, as determined by the removal of radioactivity from the supernatant fluid. The cells were then incubated at 37 °C and samples removed at intervals, cooled, washed, lysed with 1% SDS and centrifuged in sucrose gradients. As incubation proceeded an increasing amount of the radioactivity was recovered in a form that sedimented near the top of the gradient (Fig. 1). The nature of this material was investigated further by disrupting
Interaction between FMDV and pig kidney cells

Fig. 1. Sucrose gradient sedimentation of SDS extracts of PK cells which had been incubated at 37 °C with 35S-methionine-labelled virus, strain 0-1 for (a) 0, (b) 7, (c) 27 min, after attachment of virus at 0 °C.

Fig. 2. Sucrose gradient sedimentation (95000 g, 16 h) of 35S-methionine-labelled disrupted virus, strain 0-1, extracted by NP40 after incubation of virus with PK cells for 5 min at 37 °C, and 12S sub-units generated by acidification of 3H-amino acid-labelled native virus •—•, 35S; ○—○, 3H.

Fig. 3. Time course of the conversion of 35S-methionine-labelled virus, strain 0-1, to 12S sub-units by PK cells at 37 °C. ○—○, values uncorrected; •—•, values corrected for the presence of dead cells.
the cells with Nonidet and centrifuging in the presence of the same detergent. Nonidet was used because, in contrast to SDS, it does not cause breakdown of the 12S sub-unit or artificially produced 75S empty particles (Cavanagh, 1976). Sucrose gradient analysis of material extracted in this way again showed two peaks, one near the position of native virus and the second near the top of the tube, but no material sedimenting at 75S could be found.

The slowly sedimenting material co-sedimented with and had the same polypeptide composition as the 12S particles produced by acidifying $^3$H-amino acid labelled virus, that is, it contained VP1, VP2 and VP3 but not VP4 (Fig. 2, 6b). Similar results were obtained with strains of serotypes A and C of the virus.

The rate at which virus particles were converted to the 12S sub-unit was determined from the ratio of counts in the two peaks in sucrose gradients of cell lysates after different intervals at 37 °C. In calculating the results allowance was made for the proportion of dead cells which attach virus but do not degrade it (Brown et al. 1961, 1962) since the virus attaches equally well to living and dead cells (Brown et al. 1962). The results show that about 80% of the virus which attached to living cells was disrupted into 12S sub-units. Half of this alteration occurred within 10 min while 90% of the maximum disruption took place within 20 min (Fig. 3). At 24 °C the rate of disruption was five- to sixfold slower than at 37 °C and below 21 °C no disruption was detected.

---

Fig. 4. Co-sedimentation of $^{35}$S-methionine-labelled residual virus, strain o-1, with $^3$H-amino acid-labelled virus in (a) a sucrose gradient and (b) a caesium chloride gradient. ●—●, $^{35}$S; ○—○, $^3$H.
Interaction between FMDV and pig kidney cells

Fig. 5. Caesium chloride centrifugation of (a) native- (b) SDS-extracted and (c) NP40-extracted virus. PK cells were incubated with $^{35}$S-methionine- and $^3$H-uridine-labelled virus at 37 °C for 20 min. The residual virus was recovered after sedimentation of the extracts in sucrose gradients. The broad arrows indicate the boundary between the caesium chloride and a 2 ml pad of 22 % sucrose.

**Properties of the undegraded residual virus**

For convenience we have used the term residual virus to describe the cell-associated virus that was not degraded to 12S sub-units and could be recovered by treatment with either SDS or Nonidet. The residual virus examined in Fig. 4(a) was extracted after virus had been incubated with cells for 5 min at 37 °C. The sedimentation coefficient was calculated to be about 130S from a co-sedimentation experiment with $^3$H-amino acid labelled virus particles (Fig. 4a). The shoulder at about 150S contained incompletely dissociated cellular material and recycling this peak after further treatment with detergents gave two peaks at the positions of residual virus and 12S sub-units (data not shown).

After sedimentation in a sucrose gradient the fractions containing residual virus were pooled, diluted twofold and placed on a 5 ml CsCl gradient (1.2 to 1.6 g/ml) with a 2 ml intermediate layer of sucrose. The tube was centrifuged at 70000 g for 6 h. Two distinct
peaks were obtained at 1.40 and 1.30 to 1.35 g/ml compared with 1.43 g/ml for the native virus (Fig. 4b). The relative amounts of residual virus in each peak were similar in any single experiment, irrespective of the temperature at which the virus and the cells had been incubated. However, the relative amounts varied between experiments and in some experiments most of the residual virus had a density of 1.30 to 1.35 g/ml.

There was a correlation between the sedimentation coefficient and buoyant density of the residual virus in that those particles with the lowest sedimentation rate had the lowest buoyant density. Some of the slowly sedimenting residual virus had a density of 1.22 g/ml in CsCl.

Residual virus had the same relative amounts of RNA and protein as native virus (Fig. 5) irrespective of its sedimentation coefficient or buoyant density and the polypeptide composition was the same as that of native virus (Fig. 6a). The specific infectivity (infectivity/radioactivity) of the particles relative to that of the native virus generally varied in different experiments between 0.05 and 1.0, but was usually between 0.2 and 1.0 (Table 1).

Less than 8% of the virus which attached to cells at 0°C eluted spontaneously within 60 min at 37°C. The properties of this spontaneously eluting virus were similar to those of the virus extracted with detergent, except that on some occasions the spontaneously eluting

---

**Fig. 6.** Fluorograph of a polyacrylamide gel electrophoretic separation of the 35S-methionine labelled polypeptides of (a) native virus; (b) acid generated 12S sub-units; (c) 12S sub-units extracted from infected PK cells; (d) 12S sub-units extracted from PK cells treated with trypsin before disruption of the cells; (e) 12S sub-units, extracted from cells, which had been treated with trypsin after disruption of the cells.
virus had a density of about 1.20 g/ml in CsCl. Treatment of this material with ether increased its buoyant density to 1.35 g/ml, indicating that the low density material contained lipid.

**Cellular location of disrupted virus**

The cellular location of the 12S sub-units was determined by making use of the susceptibility of the virus polypeptide VP1 to tryptic digestion (Wild et al. 1969). PK cells were incubated with $^{35}$S-methionine labelled virus at 37 °C for 20 min and the mixture then cooled to 0 °C. The suspension was divided into three equal portions which were treated as follows: (1) and (2) were incubated with PBS alone; (3) was incubated with PBS containing 2 mg/ml trypsin at 0 °C for 10 min, washed three times with PBS containing 10% bovine serum at 0 °C and suspended in PBS containing 5 mg/ml phenyl methyl sulphonyl fluoride (an inhibitor of proteolytic enzymes). All three samples were then sonicated for 5 s to disrupt the cells. Trypsin (2 mg/ml was added to portion (2), followed 10 min later by phenyl methyl sulphonyl fluoride (5 mg/ml). The three samples were then centrifuged in 5 to 20% sucrose gradients for 16 h at 90,000 g. A fourth gradient was used for $^{35}$S-methionine labelled virus that had been converted to 1aS sub-units by treatment at pH 6.5. Each gradient had a peak of radioactivity at about 12S. These peaks were precipitated with 2 vol. acetone in the presence of 50 μg bovine plasma albumin and analysed by polyacrylamide gel electrophoresis. The pattern obtained for the 12S sub-unit obtained from cells was similar to that obtained by acid disruption of the virus, regardless of whether the cells had been treated with trypsin before disruption, i.e. (1) and (3) above. Each pattern contained similar relative amounts of VP1, VP2 and VP3 but lacked VP4 (VP2 and VP3 did not separate on the gel). However, treatment of the cells with trypsin after sonication [sample (2)] resulted in the cleavage of VP1 to produce two bands not found in the 12S preparation (Fig. 6). These results indicate that most of the disrupted virus is located within the cells.

Only traces of the 12S sub-unit were found in the supernatant of cells after incubation at 37 °C, indicating that little disruption of the virus had occurred at the cell surface, with subsequent elution of 12S sub-units.
DISCUSSION

FMDV attached to PK cells at 0 °C and can only be removed in a form which is modified with respect to its sedimentation coefficient and buoyant density. The modified particles generally have a specific infectivity between 20 and 100% of that of the native virus and they contain all the virus RNA and polypeptides. The modification is probably caused by association with cellular components which cannot be removed completely with SDS or Nonidet. However, attempts to label the cellular component(s) associated with the modified virus particles, either by using cells pre-labelled with radioactive amino acids and 32P or by iodinating the isolated complex have been unsuccessful. This situation is similar to that described by Lonberg-Holm et al. (1975) who found that poliovirus recovered from HeLa cells infected in the presence of a methylthiopyrimidine S7, a compound which allows attachment but prevents uncoating of poliovirus by HeLa cells, was associated with cellular components and had a reduced sedimentation coefficient and specific infectivity. However, these cellular components could be removed from the poliovirus complex by treatment with detergents.

When the FMDV-cell complex was incubated at 37 °C up to 10% of the virus was eluted and this also had a buoyant density in CsCl and specific infectivity lower than that of the native virus. The buoyant density was 1.20 g/ml but this increased to 1.35 g/ml after treatment with ether, suggesting that cellular lipid components were associated with the eluted virus. Several other picornaviruses also spontaneously elute from susceptible cells at 37 °C in association with cellular components (Joklik & Darnell, 1961; Fenwick & Cooper, 1962; Crowell & Philipson, 1971; Lonberg-Holm & Philipson, 1974; Noble-Harvey & Lonberg-Holm, 1974) but with these viruses alteration of the capsid structure had occurred involving the loss of VP4 and resulting in the instability of the particles in CsCl.

Of the FMDV remaining associated with PK cells at 37 °C, 20% was undegraded and could be recovered as a complex with cellular components by detergent treatment. This residual virus had properties similar to that recovered at 0 °C, namely reduced infectivity, sedimentation coefficient and buoyant density. However, it contained all the virus RNA and polypeptides and the virus capsid was stable during CsCl centrifugation. This is in contrast to the situation with other picornaviruses where virus recovered by detergent treatment of the virus-cell complex had very low infectivity, lacked VP4 and was unstable in CsCl gradients.

Both the spontaneously eluting FMDV cell complex and that extracted with detergents gave three peaks in CsCl gradients at densities of 1.20, 1.30 to 1.35 and 1.40 g/ml. It seems likely that some lipid is associated with the complex of density 1.20 g/ml since ether increases its density to 1.35 g/ml. The complex with a density of 1.40 g/ml may be virus associated with the specific receptor, as suggested for adenovirus (Lonberg-Holm & Philipson, 1969), whereas that with a density of 1.30 to 1.35 g/ml could have additional cellular material associated with it.

The 80% of the virus associated with the cells which could not be recovered as intact particles was recovered as 12S sub-units, indistinguishable from those produced from virus particles by acidification (Fig. 6); there was no suggestion that VP1, 2 or 3 had been cleaved either during or following the uncoating step. No other virus related structures were found, even when we used virus of serotype A, from which empty particles can be produced readily by treatment with EDTA (Rowlands et al. 1975) or at pH 6.5 (Cavanagh, 1976). The polypeptides of the 12S sub-units were resistant to cleavage by trypsin provided that the cells were intact during the enzyme treatment suggesting that the 12S sub-units were located inside the cells.

The experiments described in this paper show that the sequence of events on adding
Interaction between FMDV and pig kidney cells

FMDV to PK cells can be divided into three stages; (1) apparently irreversible attachment of the virus to components of the cell surface without apparent destabilization of the capsid; (2) spontaneous elution of a small proportion of the attached virus in association with cellular components without apparent alteration of the polypeptide composition of the virus particle; (3) rapid penetration possibly in vacuoles and disruption of the virus into 12S sub-units. This sequence of events differs from that observed with other picornaviruses where alteration of the capsid structure involving the loss of VP4 can occur at the cell surface before penetration or pseudo-penetration in vacuoles.

The process of uncoating FMDV-RNA may be similar, however, to that described by Hall & Rueckert (1971) for the cardioviruses when these are incubated with cells under conditions which prevent the release of VP4 until the virus has penetrated the cell.

REFERENCES


*(Received 23 September 1977)*