A Comparative Chemical and Serological Study of the Full and Empty Particles of Foot-and-Mouth Disease Virus

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SUMMARY

The chemical and serological properties of the full, naturally occurring empty and artificially produced empty particles of foot-and-mouth disease virus, serotype A (subtype 10, strain 61) have been studied. The full 146 S particles comprised the virus RNA, three polypeptides (VP1 to VP3) mol. wt. about $3 \times 10^8$, one polypeptide (VP4) mol. wt. about $13.5 \times 10^3$, and a small amount of a polypeptide (VP0) mol. wt. about $43 \times 10^3$. The naturally occurring 75 S empty particles contained no RNA and much less VP1 and VP4 than were found in the full particles. However they contained a much greater proportion of VP0 than the full particles. Dialysis of purified full particles against tris-EDTA, pH 7.6, produced artificial 75 S empty particles which contained only a small amount of RNA and no VP4; otherwise the polypeptide composition was similar to that of the full particles. Immunological and serological tests showed that the full particles were antigenically similar to the naturally occurring empty particles but distinct from the artificial empty particles. The latter particles, however, had serological properties similar to those of the 12 S protein subunit of the virus. Both the full and naturally occurring empty particles attached efficiently to susceptible cells, whereas the artificial empty particles attached only to a limited extent. The results are related to the function of the individual polypeptides of the virus particle and compared with published work on other picornaviruses.

INTRODUCTION

In contrast to full (D) poliovirus particles, the empty (i.e. RNA-free or C) particles occurring in harvests and the artificial empty particles produced by heating the full particles at 56 °C or by irradiating them with u.v. light elicit the production of only low levels of neutralizing antibody (Hummeler & Hamparian, 1958; Hummeler & Tumilowicz, 1960; Hinuma, Katagiri & Aikawa, 1970; Ghendon & Yacobson, 1971). This difference in the antigenic activity of the different particles correlated with the serological differences demonstrated by complement fixation, immuno-diffusion and immune electron microscopy tests (Le Bouvier, Schwerdt & Schaffer, 1957; Mayer et al. 1957; Hummeler, Anderson & Brown, 1962). Furthermore, the D particles attach efficiently to susceptible cells whereas both the naturally occurring and artificial empty particles do not attach (Katagiri, Hinuma & Ishida, 1968). The naturally occurring empty particles contain all the polypeptides present in the full particles but two of the polypeptides (VP2 and VP4) are present as a single uncleaved molecule (VP0). The artificial empty particles differ from the full particles in not containing any RNA or polypeptide VP4. The polypeptide conferring D antigenicity on the full particle...
is likely to be VP4 (Breindl, 1971). This polypeptide is also likely to carry the attachment site in the closely related Coxsackie B3 virus (Crowell & Phillipson, 1971).

In contrast, the situation with rhino- and foot-and-mouth disease viruses is somewhat different. The naturally occurring empty particles of rhinovirus, type 2, possess D as well as C antigenicity (Lonberg-Holm & Yin, 1973) and show limited attachment to susceptible cells (Noble & Lonberg-Holm, 1973). Similarly, as we show in this paper, the naturally occurring empty particles of foot-and-mouth disease virus also possess D antigenicity and attach as efficiently as the full particles to susceptible cells. Furthermore, our previous work with foot-and-mouth disease virus has provided evidence that only polypeptide VP1 is concerned in the formation of neutralizing antibody and attachment to susceptible cells (Wild & Brown, 1967; Wild, Burroughs & Brown, 1969). Trypsin destroys both these properties but, as judged by polyacrylamide gel electrophoresis, only VP1 is cleaved. The polypeptide VP4 does not appear to be involved in either the formation of neutralizing antibody or the attachment to cells. Furthermore, the antigenic site of VP4 appears to be located internally (Talbot et al. 1973).

In this paper the polypeptide composition and antigenic properties of the full, naturally occurring empty and artificially produced empty particles of foot-and-mouth disease virus have been studied. The implications of the results are discussed in relation to the proposed model for the virus.

**METHODS**

*Virus growth and assay.* Foot-and-mouth disease virus (serological type A, subtype 10, strain 60) was grown in BHK 21 cell monolayers in Eagle's medium. The same medium was used for growing [H]-uridine labelled virus. Methionine-free Eagle's medium was used for labelling the virus with [35S]-methionine and Earle's medium when labelling with a mixture of amino acids was required. The virus was titrated by intraperitoneal inoculation of 7-day-old mice with 10-fold dilutions of the preparations.

*Purification of full and empty particles.* Virus particles were prepared by the method described by Brown & Cartwright (1963). Purification of natural and artificial empty particles is described in the Results section.

*Iodination of virus.* Purified virus in 25 µl 0.04 M-phosphate, pH 7.6, was mixed with 100 µC [125I]-Na in 5 µl solution and 100 µg chloramine-T, in 10 µl, added. After 1 min, the mixture was diluted with 1 ml 0.04 M-phosphate and the virus separated on a Sephadex G-100 column in 0.04 M-phosphate. The virus was mixed with SDS (to 1 %) and re-cycled in a sucrose gradient to ensure freedom from low mol. wt. radioactive contaminants.

*Preparation of antisera.* Hyperimmune guinea pig antiserum was obtained by intradermal inoculation of virus passaged in guinea pigs into the hind foot pads of groups of animals, followed by an intramuscular inoculation of a similar preparation of virus 12 weeks later. The animals were exsanguinated 10 days after the second inoculation. Sera were also obtained from guinea pigs which had received subcutaneous inoculations of full and empty particles, inactivated with 0.05 % acetylhydrazine (AEI) for 6 h at 37 °C or 0.05 % formaldehyde for 72 h at 20 °C followed by 0.05 % acetylthiourea for 1 h at 37 °C. These preparations were mixed with aluminium hydroxide gel prior to inoculation.

*Determination of the activity of neutralizing antibody.* Tenfold dilutions of virus were mixed with equal vol. of guinea pig sera or the separated IgG fraction. After 1 h at 20 °C, 0.03 ml vol. of the mixtures were inoculated into groups of 7-day-old mice. The difference between the 50 % end point of an experimental mixture and that of the virus alone was taken as the neutralizing activity of 0.015 ml of the serum or serum fraction.
**Serum antibody blocking activity.** Fivefold dilutions of antigen in 0.04 M-phosphate, pH 7.6, were mixed with equal vol. of 1/125 hyperimmune antiserum for 1 h at 20 °C. The mixtures were then diluted with an equal vol. of water (to give a final phosphate concentration of 0.01 M) and passed through a column of DEAE-cellulose equilibrated with 0.01 M-phosphate of pH 7.6. The unreacted IgG which passed unadsorbed through the column was tested for virus neutralizing activity. Control mixtures of phosphate buffer alone and 1/125 hyperimmune serum were examined similarly for neutralizing activity.

**Immunodiffusion tests.** The double diffusion method described by Ouchterlony (1949) was used with minor modifications. Agarose (0.85 %) in tris-HCl, pH 7.6, and containing 0.01 % sodium azide, was used and the reactions were observed for 7 days.

**Measurement of attachment to cells.** Radioactive preparations of the full and empty particles were mixed with primary pig kidney cells suspended in phosphate-buffered saline. After 15 min at 37 °C, the suspensions were centrifuged at 600 g for 5 min and the radioactivity in the supernatant fluid and cells was measured.

**Assay of radioactivity.** Samples for scintillation counting were prepared as described in detail in Talbot et al. (1973). For autoradiography the polyacrylamide gels were sliced longitudinally and the central portion dried under vacuum on to a wettable cellophane sheet (Russell & Skehel, 1972). The dried strips were placed in contact with X-ray film for an appropriate period before developing the film.

**Polyacrylamide gel electrophoresis.** The virus polypeptides were analysed in SDS gels as described by Burroughs et al. (1971).

**RESULTS**

**Purification of full and empty particles**

Full particles were prepared by the method described by Brown & Cartwright (1963). The pellet obtained by ammonium sulphate precipitation and centrifuging at 80,000 g for 1.5 h was resuspended in 0.04 M-phosphate, pH 7.6, and dispersed with 1 % SDS prior to centrifuging in a 15 to 45 % sucrose gradient.

Natural empty particles are destroyed by SDS and to a lesser extent by sodium deoxycholate. Since they are stable in Nonidet P40, however, the last step in the purification scheme for full particles was modified by mixing the resuspended virus pellet with 0.01 % pancreatic ribonuclease at 37 °C for 15 min to destroy ribosomes and then mixed with 1 % Nonidet P40 before centrifuging in a sucrose gradient. Using this purification method with virus grown in the presence of [3H]-uridine and [14C]-amino acids, the distribution of radioactivity and infectivity showed that the empty particles contained no detectable RNA (Fig. 1a). Fractions 14 and 15 contained less than 1 % of the infectivity of the full particles (Fractions 7 to 9).

The method of K. Strohmaier (personal communication) was used to prepare artificial empty particles. Purified full particles were dialysed against 0.01 % EDTA, 0.02 M-tris, pH 7.6, for 2.5 h and then separated from any residual full particles by centrifuging in a sucrose gradient (Fig. 1b). In most experiments yields of 75S particles were about 25 % based on radioactive protein recoveries. The empty particles contained 4 % of the uridine and 0.001 % of the infectivity of the full particles from which they were prepared.

The morphology of typical preparations of the three particles is shown in Fig. 2. Our preparations of artificial empty particles always appeared to be aggregated, as shown in the lower panel, but occasionally separate particles were observed (upper panel). Since the particles were isolated from the 75S region of the gradient, the aggregation must have occurred during the preparation of the samples for microscopy.
Fig. 1. Sucrose gradient sedimentation of (a) virus pellets after treatment with 1% Nonidet P40, showing the separation of full and empty particles and (b) full particles after dialysis against EDTA. ●——●, [14C]-amino acids; ○——○, [3H]-uridine.

Polypeptide composition of full and empty particles

SDS-polyacrylamide gel electrophoresis in 10% gels at pH 7.6 of the proteins of [14C]-amino acid labelled full particles gave two main peaks of radioactivity and a very small amount of a third (Fig. 3a). Using bovine serum albumin and myoglobin as internal markers, the major peaks had mol. wt. of 30 and 13.5 x 10^3 and the minor peak had a mol. wt. of 43 x 10^3. The peak with mol. wt. 43 x 10^3 consisted of VP0 and that with mol. wt. 13.5 x 10^3 consisted of VP4. Although we were unable to resolve the major peak in 10% gels, it was considered to contain the three polypeptides VP1, VP2 and VP3 found in foot-and-mouth disease virus of serotypes 0 and Asia 1. Similar difficulties in resolving this peak have also been encountered with several other strains of the virus (Talbot et al. 1973). By increasing the gel concentration to 12.5%, the band was resolved into two peaks which were readily detected by Coomassie blue staining or by autoradiography (Fig. 3b). The intensity of
Fig. 2. Electron micrographs of full, natural empty and artificial empty particles stained with 1% phosphotungstic acid.

staining of the more slowly migrating band (VP1) was about one-half that of the faster moving band (VP2 and VP3).

Following trypsin treatment of the full particles, VP0, VP1 and VP4 were unchanged but the band containing VP2 and VP3 was reduced in intensity by about 50% and additional peaks were obtained at positions corresponding to mol. wt. of 19 and 10×10^8 (Fig. 3c). Polypeptides of these sizes are also found when other serotypes of the virus are treated with trypsin. Since the enzyme removed only about 50% of the material in the VP2 and VP3 peak, it is clear that there are at least two polypeptides migrating to this position.

Polypeptides VP1, VP2 and VP3 of serotypes 0 and Asia 1 can be clearly separated, even in 10% gels, but as these serotypes produce unstable empty particles they were not used in this study. Trypsin treatment of types 0 and Asia 1 converts VP1 into polypeptides of mol. wt. 19 and 10×10^8. With the virus used in the present work, VP2 or VP3, but not VP1, is clearly the trypsin-sensitive polypeptide.

The natural and artificial empty particles gave the polypeptide profiles shown in Fig. 4. Natural empty particles gave two major peaks (VP0 and VP2+VP3). Minor peaks corresponding to VP1 and VP4 were seen on the autoradiographs but were of such a low intensity that they did not appear in the photograph. These results are in agreement with the observations made by Vande Woude, Swaney & Bachrach (1972). In contrast, artificial empty particles gave a polypeptide profile similar to that of full particles except for the absence of VP4.

The constituent polypeptides of the major peak of full and artificial empty particles were compared by using iodinated virus. More than 95% of the iodine label of full particles was associated with the major peak containing VP1, VP2 and VP3 (Fig. 5a). After trypsin treatment, most of the label was then present in the polypeptide with mol. wt. 19×10^3 (Fig. 5b). Since VP2 or VP3 is the trypsin-sensitive polypeptide, this means that only one of
these polypeptides is iodinated in the intact virus. Conversion of iodinated full particles into empty particles did not lead to any loss in specific activity, showing that the trypsin-sensitive polypeptide is retained in the empty particles. Polyacrylamide gel electrophoresis showed that the label was still associated with the polypeptide which is sensitive to trypsin (i.e. VP2 or VP3 – see Fig. 5c).

**Serological properties of the three particles**

**Immunodiffusion**

The full and natural empty particles gave a line of identity when allowed to diffuse through 0.85% agarose gels towards hyperimmune guinea pig serum. Each precipitin band crossed the band formed by the 12S subunit obtained by heating the full or natural empty particles at 56°C (Fig. 6a). This result differs in one respect from that obtained by Graves, Cowan & Trautman (1968); these authors found that the natural empty particles produced a precipitin
Full and empty particles in FMDV

Fig. 5. Polyacrylamide gel electrophoresis of the polypeptides of untreated particles, trypsin-treated particles and artificial empty particles all of which were iodinated with $^{131}$I. The gels were sliced into 1 mm sections and counted in a Packard scintillation counter: (a) virus; (b) trypsin virus; (c) artificial empty particles.

line which fused with those produced by both the full and 12S particles, although the region of fusion with the 12S particle was 'vague and did not give the impression of strict identity' (Cowan, 1973).

In contrast to the results with the natural empty particles, the precipitin line obtained with the artificial empty particles showed no relationship with the full particles but fused with the band produced by the 12S particles (Fig. 6b).

Serum blocking

In view of the identity of the full and naturally occurring empty particles in immunodiffusion tests, serum blocking tests were made to determine whether the empty particles would react with the neutralizing antibody present in hyperimmune serum. Fivefold dilutions
Fig. 6. Immunodiffusion of full, natural empty, artificial empty and 12 S protein subunit preparations with hyperimmune guinea pig serum. A, full particles; B, natural empty particles; C, natural empty particles heated at 56 °C; D, 12 S protein subunit prepared by heating full particles at 56 °C; E, artificial empty particles; F, antiserum. The faint line seen in (a) well A, bottom right, is due to spontaneous breakdown of 146 S particles to 12 S particles.

Table 1. Antibody blocking activity of full, natural empty and artificial empty particles of FMDV

<table>
<thead>
<tr>
<th>Dilution of antigen</th>
<th>Log reduction of neutralizing activity in 0.015 ml of IgG</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Fulls</td>
</tr>
<tr>
<td>1/1</td>
<td>2.2</td>
</tr>
<tr>
<td>1/5</td>
<td>2.6</td>
</tr>
<tr>
<td>1/25</td>
<td>1.3</td>
</tr>
<tr>
<td>1/125</td>
<td>Nil</td>
</tr>
</tbody>
</table>

The mixtures of particles and \( \frac{1}{15} \) hyperimmune serum were diluted twofold and filtered through DEAE-cellulose in 0.01 M-phosphate, pH 7.6. The residual neutralizing activity was measured by titration with tenfold dilutions of virus.

of the preparations were mixed with a constant dilution of antiserum and the unreacted IgG separated by filtration through DEAE-cellulose. The virus-neutralizing activity of the filtrates was then determined. The natural empty particles had the same serum blocking activity as the full particles; however, artificial empty particles absorbed much lower levels of neutralizing antibody (Table 1).

**Immunogenic activity**

Since the natural empty particles and the full particles absorbed the same amount of neutralizing antibody, their ability to elicit the production of neutralizing antibody was compared. Only low levels of neutralizing antibody were produced in guinea pigs inoculated with natural empty particles treated with 0.05 % acetylated ethyleneimine to destroy any residual infective particles (Table 2). Cowan (1973) has also shown that natural 75 S particles similarly inactivated produce much lower levels of neutralizing activity than an equal amount of the full particles.

This finding, which was unexpected in view of the results of the serum blocking test, was explained by the fact that inactivation with AEI resulted in considerable breakdown of the
Full and empty particles in FMDV

Table 2. Immunogenic activity of full, natural empty and artificial empty particles of FMDV in guinea pigs

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Method of inactivation</th>
<th>Neutralizing activity* of serum (log ID₅₀/0.015 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fulls</td>
<td>AEI alone</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>Formalin plus AEI</td>
<td>3.5</td>
</tr>
<tr>
<td>Natural empties</td>
<td>AEI alone</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>Formalin plus AEI</td>
<td>3.1</td>
</tr>
<tr>
<td>Artificial empties</td>
<td>AEI alone</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>Formalin plus AEI</td>
<td>0.7</td>
</tr>
</tbody>
</table>

* Tenfold dilutions of virus were mixed with an equal volume of serum and 0.03 ml vol. inoculated intraperitoneally into groups of 7-day-old mice. The values in the table give the difference between the 50% end points of the virus alone and virus plus serum.

Fig. 7. Sucrose gradient of sedimentation natural empty particles after inactivation with acetyleneimine (a) and after fixing with formaldehyde prior to inactivation with acetyleneimine (b).

natural empty particles into slowly sedimenting material (Fig. 7a). If the particles were first fixed with 0.05% formaldehyde at 20 °C for 72 h before inactivation of residual infectivity with 0.05% AEI, they then sedimented at 75S (Fig. 7b) and in most experiments produced as much neutralizing antibody as the full particles (Table 2). However, in a few experiments using similar conditions, only low levels of neutralizing antibody were obtained. At present we have no explanation for the variable nature of the response to the natural empty particles.

The artificial empty particles always produced low levels of neutralizing antibody, irrespective of whether they were treated with acetyleneimine or formaldehyde (Table 2).

Attachment of the particles to susceptible cells

The ability of full particles to react with neutralizing antibody or to produce this antibody when inoculated into guinea pigs appears to be related to their ability to attach to susceptible cells, since treatment with trypsin destroys all three properties while only affecting one of
Table 3. Attachment of full, natural empty and artificial empty particles to pig kidney cells

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Radioactivity attached to cells (ct/min)</th>
<th>Radioactivity remaining in supernatant fluid (ct/min)</th>
<th>Attachment (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fulls</td>
<td>14,593</td>
<td>2,319</td>
<td>86</td>
</tr>
<tr>
<td>Natural empties</td>
<td>14,521</td>
<td>2,301</td>
<td>86</td>
</tr>
<tr>
<td>Artificial empties</td>
<td>683</td>
<td>10074</td>
<td>6</td>
</tr>
</tbody>
</table>

the capsid polypeptides (Wild et al. 1969). Both full and natural empty particles attached efficiently to pig kidney cells. However, artificial empty particles attached only to a limited extent (Table 3) despite the presence of the trypsin-sensitive polypeptide.

DISCUSSION

All the picornaviruses so far examined contain three polypeptides with mol. wt. of approx. $3 \times 10^3$ and one polypeptide with a mol. wt. of approx. $10 \times 10^3$ (for list of references see Talbot & Brown, 1972). From the relative proportions of the different polypeptides, models have been suggested for the virus particle (e.g. Rueckert, Dunker & Stoltzfus, 1969; Johnston & Martin, 1971; Talbot & Brown, 1972; Philipson, Beatrice & Crowell, 1973). In the case of foot-and-mouth disease virus, the capsid has been depicted as consisting of 20 units comprising 3 molecules each of VP1, VP2 and VP3, together with 30 molecules of VP4; this latter protein may be located internally. The $12S$ subunit, which can be derived from the virus by reducing the pH below 7, comprises VP1, VP2 and VP3 in the same proportions as they are present in the virus, but does not contain any VP4 (Burroughs et al. 1971).

Trypsin treatment cleaves one of the $3 \times 10^3$ mol. wt. polypeptides into units with mol. wt. of 19 and $10 \times 10^3$ but does not affect the morphology of the virus particles. However, the treated particles no longer attach to susceptible cells and produce only low levels of neutralizing antibody in cattle or guinea pigs. These results have led to the conclusion that the immunizing antigen and cell attachment site are associated with the trypsin-sensitive polypeptide.

Natural empty particles, which contain the trypsin-sensitive polypeptide, also produce neutralizing antibody provided they are fixed with formaldehyde prior to inoculation. However, artificial empty particles and the $12S$ protein subunit, both of which contain the trypsin-sensitive polypeptide, fail to produce neutralizing antibody. Since neither of these particles contain VP4, these observations lead to the conclusion that the immunizing activity is expressed only in a structure of which VP4 is also a member, either as an individual polypeptide (in full particles) or as part of the larger polypeptide VP0 (in natural empty particles).

The situation with poliovirus is different in that neither the naturally occurring nor the artificially produced empty particles produce neutralizing antibody. Breindl (1971) showed by immunodiffusion tests that the neutralizing antibodies in poliovirus antisera attached to VP4 of the virus particles, suggesting that the immunizing antigen of the virus is located on this polypeptide. The absence of VP4 from artificial empty particles would thus provide a ready explanation for their lack of immunizing activity. However, the lack of immunizing activity of natural empty particles is more difficult to account for but it is possible that the configuration of VP4 when it forms part of the uncleaved protein VP0 prevents it from producing neutralizing antibody. If the cell attachment site of poliovirus is located on VP4,
as it appears to be in the case of the closely related enterovirus Coxsackie B3 (Crowell & Philipson, 1971), this would suggest that neutralizing antibody reacts with the cell attachment site on the virus particle.

This fundamental difference between poliovirus and foot-and-mouth disease virus demonstrates the dangers inherent in postulating a single unifying model for the picornaviruses. These dangers are emphasized by the work of Halperen, Eggers & Tamm (1964), which showed that the full and natural empty particles of Echo 12 virus were antigenically similar and attached with equal efficiency to susceptible cells. Noble & Lonberg-Holm (1973) have also shown that only 20% of the population of natural empty particles in human rhinovirus type 2 attached to susceptible cells, although there was no difference in the polypeptide compositions of the particles which attached and those which failed to attach to the cells. Clearly, more information is required before we can attempt to explain the apparent fundamental difference in the location of the antigenic and cell attachment sites of various members of the picornavirus group.

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