Further Evidence for Multiple Proteins in the Foot-and-Mouth Disease Virus Particle

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SUMMARY

Further evidence has been obtained which confirms that foot-and-mouth disease virus contains several structural proteins. By electrophoresis in urea-polyacrylamide gels, virus of type O gave six distinct bands. In sodium dodecyl sulphate-polyacrylamide gels four proteins with molecular weights of 34, 30, 26 and 13.5 x 10^3 were clearly demonstrated. When virus preparations were labelled with a single amino acid, in both sodium dodecyl sulphate-polyacrylamide and urea-polyacrylamide gel electrophoresis, the fastest migrating protein contained no arginine and only traces of cysteine. This protein also stained differently from the other bands with Coomassie Blue and was absent from the 12S protein subunit prepared by mild acid (pH 6.5) disruption of the virus. This protein was separated from the 12S subunit by sucrose gradient centrifugation and by ion exchange chromatography on Amberlite IRC-50.

INTRODUCTION

Although there is accumulating evidence that the animal picornaviruses contain several polypeptides, controversy still persists concerning the number of polypeptides in foot-and-mouth disease virus. Vande Woude & Bachrach (1968), who found multiple bands in polyacrylamide gel electrophoresis separations of disrupted virus, concluded that they were due to aggregation of a single polypeptide. We provided two pieces of evidence, however, which indicated that the bands obtained in polyacrylamide gel electrophoresis separations of disrupted virus, concluded that they were due to aggregation of a single polypeptide. We provided two pieces of evidence, however, which indicated that the bands obtained in polyacrylamide gel electrophoresis separations of disrupted virus, concluded that they were due to aggregation of a single polypeptide. 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The present paper provides additional evidence for the existence of several polypeptides in the virus and confirms that the major immunizing antigen is located on one protein.
METHODS

Viruses. The viruses were normally grown in monolayers of BHK 21 cells in Eagle's medium at 37° until the cell sheets had been destroyed. In the experiments to determine the effect of host cell on the polypeptide composition of the virus, however, monolayers of primary calf kidney cells were used. For these preparations, the cell sheets were incubated at 37° in Earle's saline containing lactalbumin hydrolysate and yeast extract. Viruses labelled with [14C] or [3H]amino acids were grown in BHK 21 cells in Earle's medium; virus labelled with [3H]uridine was grown in Eagle's medium. All the preparations were purified by the method of Brown & Cartwright (1963).

Polyacrylamide-gel electrophoresis. Two methods were used. In the first, which is referred to as the urea-polyacrylamide method, purified virus was suspended in 8 M-urea and 1% mercaptoethanol and the disrupted virus then analysed in 7.5% acrylamide gels prepared according to the method of Davis (1964) with slight modifications. The gels measured 4 × 0.7 cm. and contained 8 M-urea. A current of 2 mA/gel was passed at 200 v for 4 hr and the gels were then stained in 0.05% Coomassie Brilliant Blue in 12.5% trichloroacetic acid (Maizel, 1966).

In the second, which will be referred to as the sodium dodecyl sulphate-polyacrylamide method, labelled virus was mixed with myoglobin and bovine plasma albumin (which acted as internal standards for each run) and disrupted with 8 M-urea, 1% mercaptoethanol and 1% sodium dodecyl sulphate for 1 hr at 37°. The mixture was then dialysed at room temperature against 0.01 M-phosphate, pH 7.2, containing 0.5 M-urea, 0.1% mercaptoethanol and 0.1% sodium dodecyl sulphate. Ten% gels measuring 10 × 0.75 cm. were prepared from a mixture containing 40 parts of acrylamide to one part of methylene bis-acrylamide in 0.5 M-urea, 0.1% sodium dodecyl sulphate and 0.1 M-phosphate, pH 7.2. The electrode chambers contained 0.1% sodium dodecyl sulphate in 0.1 M-phosphate, pH 7.2, and in addition the upper (negative) chamber contained 0.4% reduced glutathione. After pre-electrophoresis for 30 min., the sample, made to 15% (w/v) with sucrose, was layered on top of the gel and electrophoresis continued for 18 hr at 5 mA/gel. The gels were then removed from the tube, stained for 16 hr with 0.25% Coomassie Brilliant Blue in 10% methyl alcohol, 9% acetic acid and de-stained in a 10% methyl alcohol, 9% acetic acid solution.

In the urea-polyacrylamide gels the pattern of staining was recorded by photography. For radioactive counting, the gels were sliced into 1 mm. lengths, each section placed in a scintillation vial and incubated with 0.5 ml. Nuclear Chicago solubilizer at 65° for 2 hr. The samples were then held at 4° for 16 hr after mixing with toluene-based scintillant before counting in a Packard scintillation counter (Bash, 1968). In the sodium dodecyl sulphate-polyacrylamide method, staining was used only to locate the positions of the standards and the gels were then sliced and counted as described above.

Isoelectric focusing. Virus dissociated with 8 M-urea in the presence of 1% mercaptoethanol was mixed with 2% ampholytes (LKB Ampholine, pH range 3 to 10) in 6 M-urea +1% mercaptoethanol and separated at 4° in a horizontal zone convection-stabilized apparatus (P. Talbot, in preparation). A potential difference of 1000 v across the electrodes was applied for 72 hr. The gradient was then fractionated and the pH of each 1 ml. fraction determined. Samples of each fraction were then precipitated with 10% trichloroacetic acid, the protein collected on glass fibre discs and the dried discs counted in toluene-based scintillant, using a Packard scintillation counter. Appropriate fractions were examined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis.
Fig. 1. Electrophoresis in 8 M-urea-polyacrylamide gels of viruses passaged in different host cells. Virus (type C, NOVILLE strain) which had been passaged five times in BHK cells (BHK 5) was cloned in BHK or calf kidney (CK) cells and each clone was then passaged once in BHK or calf kidney cells. These viruses (BHK 7 and BHK 5 CK 2) were then passaged once in BHK or calf kidney cells before purification.
RESULTS

Effect of host cell on polyacrylamide electrophoresis pattern

Electrophoresis of disrupted preparations of several different types of the virus by the urea-polyacrylamide method gave rise to at least five bands. While we were satisfied that our purified virus preparations were free from host cell constituents as judged by several criteria (Brown & Cartwright, 1963), it seemed necessary to confirm that the bands obtained by polyacrylamide gel electrophoresis were due to virus protein and were not cellular in origin. Accordingly, the type C (Noville strain) virus, which had been passaged five times in BHK 21 monolayers, was cloned in both BHK and calf kidney (CK) monolayers. A single plaque from each cell type was isolated and passaged in its respective host. These viruses,

Fig. 2. Electrophoresis in SDS-polyacrylamide gels of virus labelled with $[^{14}C]$amino acid before and after incubation with trypsin. (a) type O, strain 1; (b) type O, strain 1, plus trypsin.
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which are referred to as BHK 7 and BHK 5 CK 2, respectively, were then passaged once in BHK or calf kidney cells to give viruses BHK 8, BHK 7 CK 1, BHK 5 CK 2 BHK 1 and BHK 5 CK 3 (Fig. 1). These preparations were purified and analysed by the urea-polyacrylamide method. The patterns obtained were very similar (Fig. 1), indicating that the protein bands were of virus origin and not due to cellular contamination. The fact that the patterns obtained with type C virus grown in BHK cells (see Fig. 8) was different from that obtained with the type O strains used here is further evidence that the bands are virus-specific.

Effect of trypsin treatment of virus on polyacrylamide electrophoresis pattern

Treatment of foot-and-mouth disease virus of type O (strain 1) with trypsin reduces the infectivity and immunizing activity without affecting the morphology of the virus particles (Wild et al. 1969). Virus which had been treated with trypsin differed from untreated virus in its urea-polyacrylamide pattern only in that the rate of migration of the slowest migrating protein then increased considerably. A similar alteration in pattern was also seen with virus which had been stored at 2° for some weeks, so that occasionally patterns were observed

Fig. 3. Electrophoresis in 8 M-urea-polyacrylamide gels of six preparations of virus (type O, strain 1) grown in the presence of a mixture of [3H]arginine, aspartic acid, leucine and threonine and one of the following [14C]amino acids: (a) lysine; (b) histidine; (c) aspartic acid; (d) glutamic acid; (e) arginine; (f) cysteine. • — — •, 3H; • — •, 14C.
intermediate between those obtained with fresh virus and trypsinized virus. Untreated and trypsin-treated preparations from virus of type O were examined by the sodium dodecyl sulphate-polyacrylamide method. In these experiments, four peaks were obtained, corresponding to proteins with molecular weights of 34, 30, 26 and $13.5 \times 10^3$. The protein of molecular weight $34 \times 10^3$ present in the untreated virus was converted to a component of molecular weight $19 \times 10^3$ and other smaller peptides by the enzyme treatment (Fig. 2a, b). The other proteins in the virus were unaffected by the treatment.

![Figure 4](image-url)

Fig. 4. Electrophoresis in sodium dodecyl sulphate-polyacrylamide gels of two preparations of virus (type O, strain 1) grown in the presence of a mixture of $[3H]$arginine, aspartic acid, leucine and threonine and one of the following $[14C]$amino acids: (a) arginine; (b) cysteine. $\cdots \cdots \cdots$, $^3H$; $\bullet \bullet \bullet \bullet$, $^{14}C$.

**Polyacrylamide electrophoresis of virus labelled with single amino acids**

Virus of type O (strain 1) was grown in the presence of a single $[14C]$amino acid and a mixture of $[3H]$amino acids containing arginine, aspartic acid, leucine and threonine. The virus was then purified and analysed by urea-polyacrylamide electrophoresis. The distribution of radioactivity in the gels was determined when the $[14C]$amino acid was (a) lysine, (b) histidine, (c) aspartic acid, (d) glutamic acid, (e) arginine or (f) cysteine (Fig. 3). Despite the lack of resolution of radioactivity in the slow-moving bands the protein which migrated the greatest distance clearly contained no arginine, only traces of cysteine and a small amount of lysine; and clearly had a different composition from the other polypeptides of the virus.

Virus preparations labelled with the same mixture of $[3H]$amino acids and $[14C]$arginine or $[14C]$cysteine were also analysed by the sodium dodecyl sulphate-polyacrylamide method. In this method also, the fastest migrating protein did not contain any $[14C]$radioactivity when virus labelled with $[14C]$arginine was used and only a trace when $[14C]$cysteine virus was used (Fig. 4). This experiment incidentally provided evidence for the identity of the fastest migrating protein in the urea-polyacrylamide and sodium dodecyl sulphate-polyacrylamide methods. In all the electrophoresis experiments, virus particles were disrupted without prior separation of the virus protein. To test whether any of the bands contained RNA, preparations of virus labelled with $[3H]$uridine and $[14C]$amino acids were analysed by the urea-polyacrylamide method. $[3H]$radioactivity was never found associated with the protein bands in the gels.
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![Graph showing isoelectric focusing profiles of viruses labeled with [14C]amino acid after disruption with 8 M-urea, 1% mercaptoethanol and separation in Ampholine (pH 3 to 10) in 6 M-urea-1% mercaptoethanol. (a) Type O, strain 1; (b) Type C, strain 997; (c) Type Asia 1, strain Israel 363. •—•, 14C; ○—○, pH.]

Coomassie Blue staining of gels

In all our experiments using the urea-polyacrylamide gel method for separating the virus proteins we noticed that the colour of the fast migrating protein was different from that of the other bands. This protein always stained purple-blue, whereas the others stained blue. While the reason for this different staining property is not known, it nevertheless provides evidence that the fast migrating protein is different from the other polypeptides.
Fig. 6. Electrophoresis in sodium dodecyl sulphate-polyacrylamide gels of fractions isolated by isoelectric focussing of [\(^{14}\)C]virus (type O, strain 1) disrupted by 8 M-urea. The fractions examined were (a) 10; (b) 14; (c) 23, from Fig. 5(a). These were mixed with virus labelled with \([^{3}\)H]amino acid before electrophoresis. ---, \(^{3}\)H; ••••, \(^{14}\)C.

Fig. 7. Sucrose gradient centrifugation of virus (type O, strain 1) labelled with \([^{14}\)C]amino acid which had been made to pH 6.5. The samples were centrifuged in the SW 25.1 rotor for 15 hr at 25,000 rev./min. in 15 to 25 % sucrose gradients prepared in 0.04 M-phosphate, pH 7.6. In (a) the virus was adjusted to pH 6.5 by adding 2 vol. 0.05 M-NaH\(_2\)PO\(_4\). In (b) the virus was mixed with 1 % bovine plasma albumin and made to 0.1 % sodium dodecyl sulphate before adjusting to pH 6.5.
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Fig. 8. Electrophoresis in 8 M-urea polyacrylamide gels of fractions obtained from virus of type O (strain 1) by disruption at pH 6·5. (a) Intact virus; (b) 12S component; (c) pellet.
Isoelectric focussing of disrupted virus

The charge-separation profiles of strains of three immunological types of virus labelled with $[^{14}\text{C}]$amino acid were significantly different (Fig. 5). Fractions from different pH zones obtained by isoelectric focusing of type O (strain 1) virus labelled with $[^{14}\text{C}]$ were mixed with virus labelled with $[^{3}\text{H}]$amino acid and analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The three major polypeptides of this virus migrated to different pH zones during isoelectric focussing, indicating that they had different isoelectric points (Fig. 6).

Disruption of virus at pH 6·5

By reducing the pH of virus suspensions to 6·5, the infectivity is reduced by about 3 logs and the RNA is released in an infectious form (Mussgay, 1959; Brown & Cartwright, 1961). It had been thought that the entire protein content of the virus was present in a $12S$ component. Sucrose gradient centrifugation of virus labelled with $[^{14}\text{C}]$amino acid (type O, strain 1 which had been reduced to pH 6·5) gave only one peak of radioactivity (Fig. 7). Closer examination of the fractions revealed, however, that about 20% of the radioactivity placed on the sucrose gradient could not be accounted for in the fractions; the missing 20% was recovered by washing the bottom of the tube with 8 M-urea + 1% mercaptoethanol solution. Polyacrylamide gel electrophoresis of the $12S$ component and pellet showed a clear qualitative difference between the two fractions (Fig. 8 and 9). The fast migrating polypeptide of the intact virus was absent from the $12S$ component. In contrast, the pellet contained all the fast migrating component together with small amounts of the other polypeptides. We considered that the presence of these polypeptides in the pellet was due to a small proportion of the $12S$ component being carried down in the aggregate, since the extent of the contamination varied between experiments.

In attempts to prevent aggregation of the fast-migrating protein on its release, virus was disrupted in the presence of bovine plasma albumin and sodium dodecyl sulphate. Difficulties were encountered in formulating the correct conditions because the $12S$ component itself was unstable in 0·1% sodium dodecyl sulphate in the absence of added protein. In some
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experiments with virus containing sufficient \[^{14}C\]amino acid radioactivity to allow the detection of the products of disruption in sucrose gradients, we were able to disrupt the virus without marked destruction of the 12S component and, at the same time, greatly reduce aggregation of the fast-migrating protein (Fig. 7b). The 12S component and the radioactive peak from the top of the sucrose gradient were examined by urea-polyacrylamide gel electrophoresis. The fast-migrating protein was absent from the 12S component but was present in the slow-sedimenting peak. The possibility that small amounts of the other virus proteins were present in the slow-sedimenting peak could not be ruled out, however, because of the presence of bovine plasma albumin in this peak.

Acid-disrupted virus was fractionated on the carboxylic acid resin Amberlite IRC-50. When the resin was equilibrated with 0.02 M-phosphate, pH 6.8, all the RNA and about 10% of the protein passed through unadsorbed. The remainder of the protein was eluted by increasing the concentration of the phosphate buffer solution to 0.2 M. Analysis of the two fractions by urea-polyacrylamide gel electrophoresis showed that the material unadsorbed at 0.02 M-phosphate contained predominantly the fast-migrating protein and the material eluted at 0.2 M contained the remaining polypeptides. Although the RNA and fast-migrating protein were not adsorbed by the resin under the conditions used here, we have no evidence that the two components are linked in the acid-disrupted preparations.

**DISCUSSION**

The experiments we describe provide evidence that foot-and-mouth disease virus contains several polypeptides. Six bands were obtained by urea-polyacrylamide gel electrophoresis of virus of type O (strain 1) whereas only four bands were obtained by the sodium dodecyl sulphate-polyacrylamide method. The molecular weights of the four bands in the sodium dodecyl sulphate system were estimated as 34, 30, 26 and 13.5 x 10^3, using myoglobin and bovine plasma albumin as internal standards. More bands were obtained by the urea-polyacrylamide system probably because it can resolve proteins which have the same size but different charge. We recognize that the urea-polyacrylamide gel method of analysis has certain drawbacks, particularly with regard to the amount of protein which does not enter the small pore gel. Nevertheless, in our hands the patterns obtained with the urea-polyacrylamide system have been reproducible for any one strain of virus, showing that this method has considerable value as a qualitative method.

We showed previously by the urea-polyacrylamide gel electrophoresis method that only one polypeptide of virus of type O (strain 1) was affected by trypsin treatment of the intact virus (Wild *et al.* 1969). This observation has been confirmed with the sodium dodecyl sulphate-polyacrylamide gel method. Only the protein with molecular weight 34 x 10^3 is affected by the enzyme, being converted to a protein with molecular weight 19 x 10^3 and smaller peptides.

By the use of virus preparations labelled with single amino acids it has been shown that the protein with molecular weight 13.5 x 10^3 contains no arginine, only a trace of cysteine and a small amount of lysine, whereas the other proteins of the virus contained these amino acids. These observations point to the distinct nature of the smaller molecular weight protein and this is emphasized by its absence from the 12S protein subunit. The absence of the small protein from the 12S component of foot-and-mouth disease virus is comparable to the observations of Rueckert, Dunker & Stoltzfus (1969) and O'Callaghan, Mak & Colter (1970) that the 14 to 15S protein subunit obtained from mouse-Elberfeld virus by similar gentle acid disruption also lacks one of the proteins present in the intact virus particle.
It is apparent from our work that foot-and-mouth disease virus, in common with other animal picornaviruses, contains several polypeptides. The role of one of the polypeptides in the function of the virus has already been established. The possible role of the small molecular weight protein in the virus is discussed in a subsequent paper (Rowlands, Burroughs, Sangar, Talbot & Brown, in preparation).

REFERENCES


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