Evidence for a Group Protein in Foot-and-Mouth Disease Virus Particles

By P. TALBOT, D. J. ROWLANDS, J. N. BURROUGHS, D. V. SANGAR AND F. BROWN

Animal Virus Research Institute, Pirbright, Woking, Surrey, England

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SUMMARY

The polypeptides of several strains of the seven serotypes of foot-and-mouth disease virus have been examined by polyacrylamide gel electrophoresis. Most strains gave a distinctive pattern of separation in urea-polyacrylamide gels but all the viruses contained one polypeptide which migrated to the same position. The mol. wt. of this polypeptide (VP 4) was shown by co-electrophoresis in sodium dodecyl sulphate-polyacrylamide gels to be the same, 13.5 × 10^3, for all seven serotypes. Since VP 4 aggregates when it is dissociated from the virus, it can be separated readily from acid-disrupted virus particles by centrifuging. It has the properties of a group antigen since it reacts in complement fixation tests with both homotypic and heterotypic antisera. The reaction between VP 4 and heterotypic antisera has also been demonstrated by using [125I]-Fab fragments. The antigenic site of VP 4 is not located on the surface of virus particles since there is no reaction between intact particles and [125I]-labelled heterotypic IgG or its Fab fragments.

INTRODUCTION

Intact particles of foot-and-mouth disease virus react only with type-specific antiserum in complement fixation and immunodiffusion tests. The mixture of RNA, 12 S protein component (comprising the three polypeptides VP 1, VP 2, VP 3) and the small mol. wt. polypeptide VP 4, produced by disruption of the virus particles at pH 6.5 or at 56 °C or by 1.5 M-guanidine, reacts also with heterotypic antiserum (Rowlands, Cartwright & Brown, 1969). In 1964 Van Oss, Dhennin & Dhennin showed that the 12 S sub-unit present in the unfractionated harvests of serotypes O and A gave lines of identity in immunodiffusion tests and more recently Cowan & Trautman (1967) demonstrated that the 12 S sub-unit isolated from virus of serotype A fixed complement with heterotypic antisera. We have confirmed these observations with purified 12 S sub-unit prepared from a range of viruses of different serotypes (D. J. Rowlands & D. V. Sangar, personal communication). In this paper we present evidence that VP 4, the polypeptide not present in the 12 S sub-unit, also cross-reacts in complement fixation tests and reacts with the Fab fraction of heterotypic antisera. The location of VP 4 in the virus particle is also discussed.

METHODS

Viruses. Strains of all seven types of the virus were grown in BHK 21 cell monolayers and purified according to the method described by Brown & Cartwright (1963).
Antisera. The sera from hyperimmunized guinea pigs were obtained from the World Reference Laboratory in this Institute. They were routinely produced by intradermal inoculation of virus passaged in guinea pigs into the hind foot pads of groups of animals, followed by an intramuscular inoculation of the same virus 12 weeks later.

Complement fixation tests. The antigens were allowed to react with excess antibody overnight at 4 °C in the presence of serial dilutions of complement (1/30 guinea-pig serum). The indicator system of sheep red blood cells and haemolysin was then added and the mixture incubated at 37 °C for 30 min.

Iodination of virus. Purified preparations of virus or trypsin treated virus (containing c. 250 μg in 0.25 ml) were mixed in rapid succession with 500 μCi: [125I]-Na (in 50 μl), 50 μg Calbiochem-lactoperoxidase (in 10 μl) and H2O2 (10 μl of 0.88 mM) at room temperature (Marchalonis, Cone & Santer, 1971). After 5 min the mixture was filtered through Sephadex G-100 (25 × 1 cm in 0.04 M-phosphate) to remove most of the excess [125I] and collected into bottles containing 0.1 ml of 0.1 % bovine plasma albumin solution. The fractions containing the iodinated virus were mixed with sodium dodecyl sulphate (1 % final concentration) and centrifuged in a 15 to 45 % sucrose gradient for 2 h at 30 000 rev/min. The treatment with sodium dodecyl sulphate was found to be necessary to remove residual [125I] which was not covalently linked to the virus protein. The [125I]-virus was collected into bottles containing 0.1 ml of 0.1 % bovine plasma albumin solution.

Iodination of IgG. Hyperimmune guinea pig serum (0.5 ml which had been dialysed against 0.01 M-phosphate, pH 7.6) was filtered through DEAE-cellulose (10 × 1 cm column). The unadsorbed γ-globulin was iodinated exactly as described for the virus and then freed from [125I]-Na by passing through a second DEAE-cellulose column. Analysis of a portion of the eluate by chromatography on Sephadex G-100 confirmed that the preparation contained no residual unreacted [125I]-Na. The remainder was centrifuged in a 15 to 25 % sucrose gradient for 16 h at 30 000 rev/min and the fractions from the 7 S region of the gradient were pooled.

Preparation of Fab from [125I]-IgG. The [125I]-IgG preparation obtained by sucrose gradient sedimentation was hydrolysed with papain in the presence of EDTA and cysteine by the method described by Cebra et al (1961), using insolubilized papain (Miles Seravac). A portion of the reaction mixture was filtered through Sephadex G-200 to check that the hydrolysis was complete. No attempt was made to separate the Fab and Fc fractions. The remainder of the reaction mixture was centrifuged for 16 h at 30 000 rev/min in a 15 to 25 % sucrose gradient and the fractions from the 3 to 4 S region of the gradient were combined.

Polyacrylamide gel electrophoresis of virus polypeptides. The methods used are described in detail by Burroughs et al. (1971).

RESULTS

Polyacrylamide gel electrophoresis of different immunological types

At least one strain from each of the seven serotypes of the virus was examined by the urea-polyacrylamide method. Examples of the staining patterns obtained with strains of each type are shown in Fig. 1. The pattern of separation of the major bands showed that clear differences exist between most of the types. The significance of the minor bands is not understood.

A striking feature of the patterns from the different strains was that the most rapidly migrating protein stained purple-blue rather than blue with Coomassie Brilliant Blue and travelled to the same position in the gels. Co-electrophoresis of mixtures of viruses confirmed that this protein was present in all the virus preparations examined (Fig. 2). In
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Fig. 1. Electrophoresis in urea-polyacrylamide gels of the proteins from strains of the seven distinct immunological types of foot-and-mouth disease virus. (a) O (strain 1); (b) A (strain 61); (c) C (strain 997); (d) Asia 1 (strain ISRAEL 3/63); (e) SAT 1 (strain 1/66); (f) SAT 2 (strain 3/57); (g) SAT 3 (strain BEC 1/65).

Fig. 2. Electrophoresis in urea-polyacrylamide gels of mixtures of viruses of types O, A, C and SAT 2. (a) O; (b) O+C; (c) C; (d) SAT 2; (e) SAT 2+A; (f) A. The virus strains were the same as in Fig. 1.
Fig. 3. Co-electrophoresis in SDS-polyacrylamide gels of [14C]-amino acid labelled type C (strain 997) virus and [3H]-amino acid labelled type O (strain 1) virus. ○—○, [14C]; ○ - - ○, [3H].

Table 1. Comparison of experimental and literature values for the mol. wts. of the proteins of several picornaviruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>Protein mol. wt. x 10^-3</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foot-and-mouth disease virus type O</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(strain 1)</td>
<td>34 (—)</td>
<td></td>
</tr>
<tr>
<td>Encephalomyocarditis virus</td>
<td>33 (34)</td>
<td>a</td>
</tr>
<tr>
<td>Human rhinovirus (type 2, strain HGP)</td>
<td>35 (36)</td>
<td>b</td>
</tr>
<tr>
<td>Bovine enterovirus (VG-5-27)</td>
<td>36 (34)</td>
<td>c</td>
</tr>
<tr>
<td>Porcine enterovirus (It 1/66)</td>
<td>35 (—)</td>
<td></td>
</tr>
</tbody>
</table>

Each virus was co-electrophoresed with foot-and-mouth disease virus, type O (strain 1) with bovine plasma albumin and myoglobin as additional internal markers. The experimental mol. wt. determinations are relative to those previously determined for the proteins of foot-and-mouth disease virus, type O. The literature values are given in parentheses. a, Butterworth et al. (1971); b, Stott & Killington (1973); c, Johnston & Martin (1971).

contrast, those bands which were typical of one strain did not necessarily coincide with bands which were typical of the second strain. Two examples are shown in Fig. 2.

A similar examination of more than 50 strains has been made in sodium dodecyl sulphate-polyacrylamide gels. With this method two basic patterns of separation were obtained. In the first, typified by viruses of the O and Asia 1 serotypes, four distinct peaks were obtained at positions corresponding to mol. wts. of 34 (VP 1), 30 (VP 2), 26 (VP 3) and 13·5 (VP 4) x 10^3 (Fig. 3). In the second group, consisting of types A, C, SAT 1, SAT 2 and SAT 3, all the strains so far examined with the exception of strain 227/66 (type SAT 2) gave two peaks (Fig. 3). The position of the larger of these peaks corresponded to a mol. wt. of 30 x 10^3 but
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Fig. 4. Co-electrophoresis in SDS-polyacrylamide gels of [14C]-amino acid labelled type C (strain 997) virus and [3H]-amino acid labelled trypsin-treated virus of the same strain. ● — ●, [14C]; ○ — ○, [3H].

evidence obtained from the examination of the trypsin-treated virus particles indicates that the larger peak is composed of at least two polypeptides (Fig. 4), the larger of which is sensitive to trypsin. The position of the smaller peak of the A, C, SAT 1, SAT 2, SAT 3 group of viruses corresponded to a mol. wt. of $1.5 \times 10^3$, which is the same as that obtained for VP 4 of types O and Asia 1 (Fig. 3). Confirmation that VP 4 in each serotype had the same mol. wt. was obtained from co-electrophoresis experiments with the polypeptides from strains of each serotype labelled with [14C]-amino acids and [3H]-amino acid labelled type O virus. No differences were found in the migration of the VP 4 polypeptides from the seven serotypes. An example of one co-electrophoresis experiment with serotypes O and C is shown in Fig. 3.

To check that the method of analysis used in these experiments would resolve polypeptides of different mol. wts. in this size range, several picornaviruses for which VP 4 has been reported as having a mol. wt. different from that of the VP 4 from foot-and-mouth disease virus were examined. Co-electrophoresis experiments with mixtures of the polypeptides from foot-and-mouth disease virus, encephalomyocarditis virus, a human rhinovirus strain HGP, bovine enterovirus VG-5-27 (Martin, Johnston & Clements, 1970) and a porcine enterovirus It 1/66 (Nardelli et al. 1968) showed that the mol. wt. of VP 4 from foot-and-mouth disease virus differed from those of the other viruses (Table I). In these experiments good resolution was obtained in this low mol. wt. range. As the peaks of VP 4 from the different foot-and-mouth disease virus serotypes coincided, the inference is that these polypeptides have the same mol. wt.

Preparation of VP 4 for serological examination

In a previous paper (Burroughs et al. 1971) we showed that disruption at pH 6.5 of virus of type O (strain 1) released virus RNA, the 12 S protein sub-unit and a polypeptide which aggregated rapidly. The aggregate was deposited at the bottom of the tube as a pellet when the disrupted virus was centrifuged for 16 h at 25000 rev/min in a 15 to 25% sucrose
Fig. 5. Electrophoresis in urea-polyacrylamide gels of the 12 S and pellet fractions of pH 6.5-
disrupted type C and type SAT 2 viruses. (a) C virus; (b) C 12 S; (c) C pellet; (d) SAT 2 virus;
(e) SAT 2 12 S; (f) SAT 2 pellet. The virus strains were the same as in Fig. 1.

gradient, i.e. under conditions necessary to band the 12 S sub-unit. Electrophoresis of the
12 S sub-unit and pellet fractions in both the urea – and sodium dodecyl sulphate – poly-
acrylamide gels showed that the 12 S sub-unit was composed of VP 1, VP 2 and VP 3
whereas the pellet contained mainly VP 4. Similar results were obtained with other types
of the virus, indicating that aggregation of VP 4 is a property of the entire group of foot-and-
mouth disease viruses (Figs. 5, 6). The small amounts of VP 1, VP 2 and VP 3 present in all
the preparations of the aggregated VP 4 were probably due to non-specific co-sedimentation
of the 12 S protein sub-unit.

Serological evidence for a group antigen on VP 4

Preparations of VP 4, made by dispersing in 2 M-urea the pellets obtained by sucrose
gradient sedimentation as described in the preceding section, fixed complement with all the
type antisera. The amounts of complement fixed were low but were similar with all the
antisera whereas the cross-reaction associated with the 12 S sub-unit was variable in extent,
depending on the particular virus and serum combination used. This suggested that the
cross-reaction obtained with VP 4 was not due to contaminating 12 S particles and further
evidence was obtained from the demonstration that hyperimmune antiserum which had
been absorbed with excess virus and 12 S particles still fixed complement with disrupted
virus.

Since the levels of complement fixing activity were low, more convincing evidence was
sought from experiments with [125I]-labelled antibody. Mixtures of excess virus or disrupted
virus and labelled IgG or Fab were allowed to react for 2 h at room temperature in the
presence of 0.02 M-EDTA. The EDTA eliminates any spurious non-specific reaction due to
complement (Maurer & Weigle, 1953). Preliminary sedimentation experiments with mixtures
of excess virus and [125I]-IgG gave two peaks of radioactivity in sucrose gradients. One peak
corresponded to the position of virus alone and the second, which sedimented faster, was presumably due to complexes containing more than one virus particle. To avoid this complex formation, \(^{125}\text{I}\)-labelled Fab was used in subsequent experiments, since mixtures of excess virus and \(^{125}\text{I}\)-Fab gave a single peak in sucrose gradients at the same position as virus alone.

Mixtures of \(^{125}\text{I}\)-Fab from type O antiserum and viruses of serotypes O, A, C and Asia 1 were centrifuged for 2 h at 30,000 rev/min in 15 to 45 % sucrose gradients. Only the homologous virus combined with the Fab fraction (Fig. 7). However, mixtures of the Fab preparation and acid or heat disrupted virus, irrespective of serotype, gave a pellet containing \(^{125}\text{I}\) after centrifuging for 16 h at 30,000 rev/min in a 15 to 25 % gradient (Fig. 8). Since VP 4 is the major virus constituent in the pellet under these conditions, the presence of radioactivity in the pellet provides evidence that VP 4 reacted with the \(^{125}\text{I}\)-Fab. The peaks of radioactivity

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**Fig. 6.** SDS-polyacrylamide gel electrophoresis of \(^{14}\text{C}\)-amino acid labelled virus, the 12 S sub-unit and the pellet recovered from the bottom of the centrifuge tube after sucrose density gradient sedimentation of acidified virus. (a) type C (strain 997); virus; (b) type O (strain 1) virus; (c) type C (strain 997) 12 S sub-unit; (d) type O (strain 1) 12 S sub-unit; (e) type C (strain 997) pellet; (f) type O (strain 997) pellet. In type C the major proteins are not resolved. The small peaks at fraction 58 in (a) and (c) are due to partial degradation of one of the major proteins.
near the top of the tube contained unreacted Fab (fractions 14 to 18) and 12 S-Fab complex (fractions 7 to 11). A control mixture of purified 12 S particles and [125I]-Fab did not give a radioactive pellet under the same sedimentation conditions, but gave a well-defined radioactive peak at the same position as 12 S particles alone (Fig. 9a).

The [125I]-Fab fraction prepared from antiserum which had first been absorbed with excess 12 S particles also reacted with VP 4 from acid-disrupted heterotypic virus (Fig. 9b), confirming that the cross-reacting site of VP 4 is not the same as that on 12 S particles. The experiment also showed that the radioactive pellet obtained with disrupted virus and heterotypic Fab is not due to reaction with contaminating 12 S polypeptides found in preparations of VP 4 obtained by centrifuging.
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Fig. 8. Sucrose density gradient sedimentation of the same strains of virus used in Fig. 7 but in this case reacted with $[^{125}\text{I}]-\text{Fab}$ derived from type O (strain 1) antibody after acid disruption of each virus. (a) type O (strain 1); (b) type A (strain 61); (c) type C (strain 997); (d) type Asia 1 (strain PAK 1/54).

Location of VP 4 in the virus particle

Since the Fab fraction of type O antiserum did not react with virus of serotypes A, C and Asia 1 (Fig. 7) the antigenic site of VP 4 is masked in the intact virus particles. Even after treatment of virus particles with trypsin, which removes part of VP 1, no reaction could be demonstrated between Asia 1 virus and type O Fab.

Using a thermolabile mutant of foot-and-mouth disease virus of serotype O, Laporte (1971) showed that only the trypsin-sensitive polypeptide was iodinated with $[^{125}\text{I}]-\text{Cl}$. When we used lactoperoxidase and $[^{125}\text{I}]-\text{Na}$ for iodinating another strain of the same serotype, more than 95% of the label was located on VP 1, the polypeptide sensitive to trypsin treatment. However, in our experiments there was a small but significant amount of label associated with VP 3 (Fig. 10a). Confirmation of the identity of the polypeptide carrying most of the $[^{125}\text{I}]$ was obtained by showing that trypsin treatment of the virus converted the
iodinated VP 1 into a labelled polypeptide with a mol. wt. of $19 \times 10^3$ (Fig. 10c). When the virus was iodinated with [$^{125}$I]-Na plus lactoperoxidase after trypsin treatment, polypeptide VP 4 was also iodinated in addition to VP 3 and the residual portion of VP 1 (Fig. 10b). This result suggests that those amino acids of VP 4 which are iodinated under these conditions are masked by VP 1 in the intact virus.

**DISCUSSION**

Infective (140 S) foot-and-mouth disease virus particles fix complement with type-specific antiserum but not with antisera produced by inoculation with the remaining six serotypes of the virus. By disrupting the virus at pH 6.5 or at 56 °C or with 1.5 M-guanidine (Rowlands, et al. 1969) cross-reactions are then observed with heterotypic antisera in complement fixation and immunodiffusion tests. The three methods of disruption release the RNA as an infective molecule and the four virus polypeptides are then observed as two distinct entities, the smaller mol. wt. polypeptide VP 4 and a 12 S sub-unit comprising VP 1, VP 2 and VP 3. In the absence of guanidine VP 4 aggregates rapidly, thus allowing it to be separated from the 12 S sub-unit.

Van Oss et al. (1964) and Cowan & Trautman (1967) demonstrated the cross-reactivity of the 12 S sub-unit with two serotypes and we have confirmed their findings with a wide range of viruses from all seven serotypes, using both complement fixation and immunodiffusion tests (D. J. Rowlands & D. V. Sangar, personal communication). In this paper we have presented evidence for the cross-reactivity of VP 4 and shown that the cross-reacting antigen on this polypeptide differs from that on the 12 S particles. The cross-reacting antigenic site is not present on the surface of the virus, since the virus particles do not react with heterotypic antiserum. Moreover, the cross-reacting site is not exposed even when part of VP 1 is removed with trypsin although VP 4 can then be iodinated.
In contrast to our result with foot-and-mouth disease virus, Breindl (1971) has shown with the structurally similar poliovirus that VP 4 is involved in the reaction of the virus particles with neutralizing antibody. Further, Crowell & Philipson (1971) have postulated that VP 4 is involved in the attachment of Coxsackie B 3 virus to HeLa cells.

Our experiments with foot-and-mouth disease virus have demonstrated that VP 1 is the polypeptide involved in the attachment to susceptible cells (Wild & Brown, 1967; Wild, Burroughs & Brown, 1969; Rowlands, Sangar & Brown, 1971) whereas VP 4 has not been shown to have any role in this connexion. In the case of this virus, VP 4 appears to have several properties which are the same for all serotypes and to be a group antigen.

REFERENCES


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