Surface Structure of Foot-and-Mouth Disease Virus

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SUMMARY

Polyacrylamide-gel electrophoresis of foot-and-mouth disease virus disrupted with 8M-urea revealed the presence of five major protein bands and one minor one. Evidence was obtained suggesting the likelihood of these bands being due to the presence of distinct polypeptides in the virus protein. Virus which had lost 3 to 4 log. of infectivity by treatment with trypsin also yielded six bands in polyacrylamide-gel electrophoresis. Five of these bands had the same mobility and intensity as those found in untreated virus but the slowest-moving band in the untreated virus had an enhanced mobility after enzyme treatment, suggesting that only one of the constituent polypeptides of the virus protein was affected by the enzyme. This polypeptide is necessary for the attachment of the virus to susceptible cells and for the immunizing activity of the inactivated virus particle.

INTRODUCTION

Foot-and-mouth disease virus is approximately spherical, has a diameter of 25 nm. and contains single-stranded RNA. Because of its small size, electron microscopy has not provided much detailed information on the surface structure of the virus (Breese, Trautman & Bachrach, 1965). It seemed, however, that information on the surface structure could be obtained by examining the virus proteins by polyacrylamide-gel electrophoresis after treatment of the virus with reagents known to affect only the surface of the virus capsid. In an earlier report we showed that trypsin considerably reduced the infectivity of the virus without apparently affecting its gross structure (Wild & Brown, 1967). The rate of sedimentation of the virus was unaltered and the RNA within the particle was still present as an intact infective molecule. The enzyme treatment resulted, however, in a slight but significant increase in density of the particle, suggesting that some of the virus protein had been removed. The treated virus no longer attached to susceptible cells, nor did it stimulate the production of neutralizing antibodies. In the present report we extend those observations to provide information on the number of polypeptide chains comprising the virus protein, and attempt to correlate the loss of various biological activities resulting from trypsin treatment with alterations to the virus structural proteins.

METHODS

Viruses. Foot-and-mouth disease virus of type O (strain 1) was used for most of the work but a few experiments were done with virus of type A (strain 119). The virus was grown in monolayers of baby hamster kidney cells (BHK 21, clone 13: Macpherson & Stoker, 1962) in Eagle's medium. Virus containing labelled protein was grown in monolayers of cells in Earle's saline containing [3H]leucine or [14C]threonine or [14C]-
amino acid mixture. Virus containing labelled RNA was grown in monolayers of cells in Earle's saline containing 0.01 M-tris instead of phosphate plus [³²P]phosphate and 1 μg. actinomycin D/ml. Virus infectivity was measured by the plaque assay method in monolayers of BHK cells or by intraperitoneal injection of mice.

Preparation of purified 25 nm. component. The method used was that described by Brown & Cartwright (1963). After precipitating the virus from the culture medium with 50% saturated ammonium sulphate, the resuspended virus was sedimented by centrifugation at 60,000 g for 1 hr. The pellet was then treated with 1% sodium dodecyl sulphate and centrifuged at 25,000 rev./min. for 3½ hr in a 15 to 45% sucrose gradient. One ml. fractions were collected and those containing most virus were combined.

Serological methods. Immunodiffusion tests were made according to the method of Ouchterlony (1949), using 1% gels in 0.1 M-tris + HCl buffer, pH 7.6. Sodium azide was used as preservative. For the determination of complement-fixing activity, excess antiserum was used when the activity of the antigens was being measured and excess antigen when the antiserum fractions were under test. Serial dilutions of complement were used and a fixation period of 30 min. at 37°C was followed by a similar period after adding the red blood cell + haemolysin indicator system.

Serum-blocking test. The antigens were inactivated with 0.05% acetylated ethyleneimine at 37°C for 6 hr and the excess inactivating agent then neutralized with 2% sodium thiosulphate. Serial twofold dilutions of the inactivated preparations were mixed with an equal volume of 1/1000 hyperimmune guinea-pig serum and kept at room temperature for 1 hr. The mixtures were then tested for residual virus neutralizing activity by mixing samples with an equal volume of tenfold dilutions of virus and inoculating into mice. When the amount of antigen was sufficient to block the neutralizing activity of the 1/1000 serum, the mixture gave the same titration end-point as the virus alone. In the absence of blocking activity, the titre obtained was the same as that of the control mixture of virus and 1/2000 serum.

Cellulose acetate electrophoresis. Strips of 'Oxoid' cellulose acetate measuring 12 x 2.5 cm. were moistened with a solution containing 0.1 M-NaCl and 0.01 M-tris + HCl buffer, pH 7.6, and excess solution then removed with filter paper. After placing in position in an electrophoresis apparatus similar to that described by Kohn (1958), samples (5 to 10 μl.) of the virus preparations were applied as narrow bands and the current (4 mA/cm. length) passed for 3 hr. The strips were then cut longitudinally into two pieces. One half was cut laterally into 0.5 cm. sections and the separate sections placed quickly into 0.04M-phosphate, pH 7.6, containing 0.1% sodium dodecyl sulphate, before they had dried out. The eluates were used for infectivity determination. The other half of the cellulose acetate strips was allowed to dry and then counted in an EKCO chromatogram scanner (type no. N 679A).

Polyacrylamide-gel electrophoresis. Virus isolated from a sucrose gradient was suspended in 8M-urea + 1% (v/v) mercaptoethanol and analysed in 7.5% (w/v) acrylamide gels prepared according to the method of Davis (1964) with slight modifications. The gels measured 4 x 0.7 cm. and contained 8M-urea. A current of 2 mA/gel was passed at a voltage of 200 v for 4 hr. The gels were then stained in 0.05% Coomassie Brilliant Blue in 12.5% (w/v) trichloracetic acid (Maizel, 1966). For radioactive counting, the gels were cooled in a mixture of acetone + solid CO₂ and sliced with a razor blade. The slices were dissolved by heating each in 0.1 ml. 100 vol. H₂O₂ at 80°C for 4 hr and then counted in Bray's solution in a Packard Scintillation Counter no. 3310.
RESULTS

Effect of trypsin on the antigenic structure of the 25 nm. component

The infective 25 nm. component of foot-and-mouth disease virus is degraded to infective RNA and a 7 nm. protein component when heated at 56° or the pH is reduced to 6.8. When inoculated into guinea-pigs the 25 nm. component gives rise to at least two antibodies, one of which is specific for the 25 nm. component and a second which will also react with the 7 nm. component. Both antibodies are stimulated by sites on the intact virus particle (Wild, 1968). In contrast, the 7 nm. component gives rise to a specific anti-7 nm. antibody (Ceglowski, 1965; Wild, 1968).

Wild & Brown (1967) showed that trypsin affected the ability of the 25 nm. component to stimulate neutralizing antibodies when inoculated into guinea-pigs, suggesting that the antigenic structure had been modified by the enzyme. We investigated the problem further to determine whether trypsin removes a specific antigenic site on the virus surface. Treatment of the 25 nm. component did not alter its complement-fixing activity when hyperimmune guinea-pig serum was used for the assay. Cartwright (1962) showed that hyperimmune guinea-pig serum contained antibodies fixing complement with both the 25 and 7 nm. components. A test was therefore made using antiserum from which antibody against the second of these components had been removed by absorption with excess 7 nm. component. The absorbed serum, which fixed complement with the 25 nm. component but not with the 7 nm. component, was then used to test the effect of trypsin on the 25 nm. component. Even using this absorbed serum, however, we could detect no difference between the complement-fixing activities of untreated and trypsin-treated 25 nm. component. By using immunodiffusion methods, however, it was possible to detect a faint but distinct spur when the untreated and trypsin-treated preparations were diffused from adjacent wells towards the hyperimmune serum (Pl. 1a). This spur was more apparent when 19 S antibody was used. The 19 S antibody is present in guinea-pigs infected with the virus within 4 days of infection and reacts specifically with the 25 nm. component of the virus (Brown & Graves, 1959; Brown, 1960). These immunodiffusion results are consistent with the concept that trypsin removes an antigenic site from the 25 nm. component. By adding excess of trypsin-treated 25 nm. component to hyperimmune serum and then centrifuging the mixture at 30,000 rev./min. for 1 hr in the 40 rotor of the Spinco Ultracentrifuge, about 90% of the complement-fixing activity of the serum directed against the 25 nm. component was removed in the deposit. This suggested that c. 10% of the antibody was directed against sites altered or removed by the trypsin. The absorbed serum still possessed most of the original neutralizing activity and gave a precipitin line in immunodiffusion tests with the 25 nm. component. It did not give a precipitin line with the trypsin-treated component.

The failure of trypsin-treated virus to react with the neutralizing antibody in hyperimmune serum was also shown by adding dilutions of a trypsin-treated preparation of the 25 nm. component to 1/1000 hyperimmune serum and then measuring the residual neutralizing activity of the serum. (The residual virus infectivity of the trypsin-treated virus was first inactivated with 0.05% acetyleneimine for 6 hr at 37° (Brown & Crick, 1959), a procedure which does not alter the antigenic structure of the virus.) Control mixtures of the same preparation of 25 nm. component (inactivated with acetyleneimine) and 1/1000 hyperimmune serum possessed only a little neutral-
(a) Immunodiffusion analysis of foot-and-mouth disease virus showing the effect of trypsin on the antigenic structure of the 25 nm component. (A) Hyperimmune guinea-pig serum; (B) 19 S γ-globulin from infected guinea-pigs; (C) untreated virus; (T) trypsin-treated virus.

(b) Polyacrylamide-gel electrophoresis of 25 nm component disrupted by 8M-urea, showing the effect of trypsin.
izing activity whereas the neutralizing activity of the trypsin-treated virus + serum mixture was similar to that of the serum alone (Table I).

**Effect of trypsin on the physico-chemical properties of the virus**

Purified $^{32}$P-labelled 25 nm. component was examined by electrophoresis on cellulose acetate membranes in 0.1M-NaCl containing 0.01M-tris, pH 7.6. Figure 1 shows the distribution of infectivity and radioactivity on the strip after passing a current of 4 mA/cm. for 3 hr. The virus remained close to the point of application under these conditions. In contrast, trypsin-treated virus migrated 2.6 cm. in the same time (Fig. 1).

### Table 1. Effect of trypsin on the antibody-blocking activity of the 25 nm. component of foot-and-mouth disease virus

<table>
<thead>
<tr>
<th>Antigen added to serum</th>
<th>Dilution of antigen</th>
<th>Infectivity* (log. ID 50/0.03 ml.)</th>
<th>Neutralizing activity of antigen + antisera mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 nm. component</td>
<td>1/1</td>
<td>5.2</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>1/2</td>
<td>5.4</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>1/4</td>
<td>6.0</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>1/8</td>
<td>5.8</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>1/16</td>
<td>5.6</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>1/32</td>
<td>5.2</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>1/64</td>
<td>4.4</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>1/128</td>
<td>4.0</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>1/256</td>
<td>3.4</td>
<td>2.5</td>
</tr>
<tr>
<td>Trypsin-treated 25 nm. component</td>
<td>1/1</td>
<td>4.0</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>1/2</td>
<td>3.4</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>1/4</td>
<td>2.0</td>
<td>3.9</td>
</tr>
<tr>
<td>Control (equal volume of phosphate buffer)</td>
<td></td>
<td>2.2</td>
<td>3.7</td>
</tr>
</tbody>
</table>

*Virus alone had an infectivity titre of $10^4$ ID 50/0.03 ml. The end-points of the mixtures were adjusted to allow for the fourfold dilution caused by addition of the inactivated antigen and the virus.

The residual infectivity, which survived the enzyme treatment, remained near the origin. The increased migration of the virus after trypsin treatment suggested that a basic group had been removed by the enzyme. The possibility that the alteration in charge on the virus produced by the enzyme was due to the formation of a virus + enzyme complex appeared unlikely, as trypsin alone migrated a shorter distance (1.3 cm.) than the radioactive peak. A complex would be expected to have an electric charge intermediate between those of the virus and the enzyme.

To determine whether the enzyme altered a single protein or attacked a number of different sites on the surface of the virus capsid, it was necessary to examine the proteins of the virus in their monomeric state. Preparations of the 25 nm. component were disrupted with 8M-urea in the presence of 1% mercaptoethanol and then examined by electrophoresis in polyacrylamide gels. When 100 to 150 µg. of virus was used for a single run, the individual proteins could be detected by staining with Coomassie Brilliant Blue (Maizel, 1966). Most preparations of virus gave five distinct well-stained bands and one very faint band when examined in this way (Pl. 1 b). Virus which had been treated with trypsin and then separated from the enzyme by sucrose-gradient centrifugation also gave six bands but the pattern differed in one important respect from the untreated virus preparation. The slowest migrating band (VP1) in the untreated
preparation was now missing but had been replaced by a band intermediate in position between VP₅ and VP₆ (Pl. 1 b), suggesting that the enzyme attacked only one of the proteins of the virus capsid. The increased mobility of this protein was consistent with the removal of a basic group by the enzyme.

Fig. 1. Electrophoresis on cellulose acetate of the ³⁵P-labelled 25 nm. component, showing the effect of trypsin on the mobility and infectivity.

Table 2. Ratio of [³H]leucine to [¹⁴C]threonine in the individual protein bands obtained by polyacrylamide-gel electrophoresis of foot-and-mouth disease virus

<table>
<thead>
<tr>
<th>Protein band</th>
<th>Portion 1</th>
<th>Portion 2</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3·8</td>
<td>4·0</td>
<td>3·9</td>
</tr>
<tr>
<td>2</td>
<td>4·7</td>
<td>4·7</td>
<td>4·7</td>
</tr>
<tr>
<td>3</td>
<td>5·7</td>
<td>5·4</td>
<td>5·5  (5)</td>
</tr>
<tr>
<td>4</td>
<td>4·2</td>
<td>3·4</td>
<td>3·8</td>
</tr>
<tr>
<td>6</td>
<td>2·3</td>
<td>2·4</td>
<td>2·3  (5)</td>
</tr>
</tbody>
</table>

* To check the experimental procedure the individual bands were sliced transversely and each portion counted separately.

The uncertainties which have surrounded the use of polyacrylamide-gel electrophoresis for determining the number of proteins making up the virus capsid are well documented (Burness & Walter, 1967). In particular, Vande-Woude & Bachrach (1968) have argued that the multiplicity of bands obtained with foot-and-mouth disease virus is due to aggregation of a single protein. We tested this by examining a
mixture containing the proteins from virus which had been grown in the presence of either [3H]leucine or [14C]threonine. The gels were then sliced and the ratio of 3H and 14C counts in the individual bands, except for the minor VP₅, was determined. The individual bands gave ratios ranging from 2.4 to 5.5 (Table 2). As a check on the experimental procedure, individual bands were sliced transversely and each portion counted separately. The different portions of each individual band gave similar ratios, except for VP₄, but this may have been caused by contamination of the lower portion of VP₄ with VP₅ (Table 2). The above evidence supported the concept that the individual bands were not formed as a result of aggregation of a single polypeptide. Additional evidence that the protein disrupted by 8M-urea was not aggregated was obtained by centrifuging the disrupted 14C-labelled virus protein in a 5% to 25% sucrose gradient containing 8M-urea for 72 hr at 25,000 rev./min. Only one peak of radioactivity was obtained with an S value less than that of bovine plasma albumin (3.8 S).

**DISCUSSION**

We have shown previously (Wild & Brown, 1967) that the loss of infectivity observed when foot-and-mouth disease virus is incubated with trypsin is accompanied by the removal of a small amount of protein from the virus particle. The enzyme-treated virus will not attach to susceptible cells and does not stimulate production of virus-neutralizing antibodies when inoculated into guinea pigs. The RNA of the virus remains in an infective and undegraded form within the particle. In the present work we have shown by immunodiffusion analysis that the virus loses an antigenic site when incubated with trypsin. The enzyme-treated virus absorbed from hyperimmune serum 90% of the complement-fixing activity directed against the virus, without lowering the virus-neutralizing activity. It seems reasonable to assume therefore that the antigenic site removed by the trypsin is the one responsible for the stimulation of neutralizing antibody.

As expected from the knowledge that trypsin attacks peptide links adjacent to arginine or lysine residues, the treated virus has a mobility in an electric field which is markedly different from that of the untreated virus. In our experiments at pH 7.6 the untreated virus remained close to the point of application on the cellulose acetate, whereas the enzyme-treated virus moved towards the anode. We are unable to offer an explanation for the observation made by Bachrach and his colleagues that trypsin has no effect on foot-and-mouth disease virus (Vande-Woude, Trautman & Bachrach, 1967). Using virus of type A, strain 119, these authors did not observe any changes in electrophoretic mobility after treatment with trypsin. In case the observations we had made with virus of type O were due to the use of a different immunological type, we repeated some of our experiments with the same strain used by Vande-Woude, Trautman & Bachrach. With this strain also, we observed the same effect of trypsin on the electrophoretic mobility that we had seen with virus of type O.

We have attempted to determine whether trypsin attacks a specific site on the virus capsid or hydrolyses several sites on the surface of the virus particle by examining the protein of the virus before and after treatment with the enzyme. Polyacrylamide-gel electrophoresis of virus protein which had been treated with 8M-urea + 1% mercaptoethanol consistently gave five major bands and one minor band. Vande-Woude & Bachrach (1968) also obtained multiple bands with foot-and-mouth disease virus
protein but concluded that the multiplicity was due to aggregation of a single protein. In view of their conclusion, we examined a mixture of purified viruses which had been grown separately in medium containing \([\text{H}]\text{leucine or [}^{14}\text{C}]\text{threonine and measured the ratio of the two isotopes in individual bands of the gel. The ratios in the different bands were not the same, which is firm evidence against aggregation having occurred and in favour of the bands being due to distinct polypeptides. The chances of chemical modification due to oxidation by the persulphate (used in preparing the gel) or carbamylation (by the cyanate in the urea) of certain amino acids were minimized by using gels which were pre-treated with thioglycollate or electrophoresed before adding the sample or by using de-ionized urea.

The trypsin-treated virus also gave five major bands and one minor band but differed from the untreated virus in that the slowest moving band was missing but was replaced by a band which migrated between VP_6 and VP_8. This new band presumably corresponds with the slowest-moving band which has been altered by the enzyme treatment. The remaining bands were located in exactly the same positions as in the untreated virus preparation, showing that only one of the proteins of the virus is affected by the enzyme. This protein contains the cell attachment site and the immunizing antigen.

We wish to thank Dr D. N. Black for his suggestions concerning the double-labelling experiment and Mr D. Sangar for assistance with some of the polyacrylamide-gel electrophoresis experiments.

REFERENCES


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