Demonstration of Three Specific Sites on the Surface of Foot-and-Mouth Disease Virus by Antibody Complexing

By F. BROWN AND C. J. SMALE
Animal Virus Research Institute, Pirbright, Surrey

(Accepted 29 December 1969)

SUMMARY

The reactions of foot-and-mouth disease virus with IgG and IgM have been studied by agar precipitin tests and by electron microscopy. The 140S component produces a precipitin band with each antibody class. It also produces a band with IgG which has been absorbed with 12S virus protein sub-units or trypsin-treated virus, providing evidence for the presence of more than one type of combining site. Trypsin-treated virus also gives a band with IgG and with IgG absorbed with 12S protein sub-units, indicating the presence of a third site. Electron microscopy shows that IgG reacts with the entire surface of the virus particle, producing complexes in which the outline of the virus particles is obscure. In contrast, IgM or IgG which has been absorbed with 12S protein sub-units or trypsin-treated virus forms complexes in which attachment is at regularly spaced sites on the virus surface. Trypsin-treated virus also produces complexes of this type with IgG absorbed with 12S protein sub-units. The virus thus appears to possess at least three types of combining site, one on the faces of the particle and the others at regularly spaced intervals, probably at the vertices.

INTRODUCTION

Particles of foot-and-mouth disease virus react with the virus-specific IgG and IgM to form complexes which can be examined in the electron microscope (Almeida, Brown & Waterson, 1967). The complexes differ in two respects. First, the virus particles complexed with IgM are at a greater distance from each other than those in the IgG complexes because of the greater length of the IgM molecules. Second and more important, the virus particles in the IgM complexes appear to be attached to antibody at specific sites, whereas in the IgG complexes the antibody molecules are attached to the entire surface, giving the virus particles an indistinct outline. These observations suggest that IgG reacts with more than one type of combining site, whereas the IgM reacts more specifically with the virus. The experiments described in this paper show that the surface of the virus possesses at least three types of combining site.

METHODS

Virus. Two sources of type O virus were used. Purified virus particles were prepared from virus grown in BHK 21 cells, using the method described by Brown & Cartwright (1963). This virus was used for the complexing experiments and for the preparation of fractions for the absorption of antibody. For the production of antisera, virus of the same strain, passaged in guinea-pig pads, was used to infect guinea-pigs. Virus grown in the same host was also
used to titrate the virus-neutralizing activity of the antisera and IgG and IgM preparations.

**Virus fractions.** The 12s protein sub-unit of the virus was prepared either by heating purified virus at 56° for 1 hr or by reducing the pH of the virus suspension to 6-8 (Brown & Cartwright, 1961) and was not further purified. Trypsin-treated virus was prepared by incubating virus concentrates at 37° for 15 min., using an enzyme concentration of 1 mg./ml., followed by addition of 1% sodium dodecyl sulphate and sucrose gradient centrifugation. Trypsin-treated virus sediments at approximately the same rate as untreated virus in sucrose gradients (Wild & Brown, 1967).

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Log ID&lt;sub&gt;50&lt;/sub&gt; neutralized by 0.015 ml. preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control—not absorbed</td>
<td>4.0</td>
</tr>
<tr>
<td>Absorbed with 12s protein sub-units</td>
<td>3.8</td>
</tr>
<tr>
<td>Absorbed with trypsin-treated virus</td>
<td>3.4</td>
</tr>
</tbody>
</table>

0.1 ml. of hyperimmune serum was mixed with excess 12s protein sub-units or trypsin-treated virus, diluted with 0.01 M-phosphate and then filtered through DEAE-cellulose. The IgG was collected in 5 ml., representing a 50-fold dilution of the serum.

**Antisera and antibody preparations.** Antibody of the IgG class was prepared from the serum of guinea-pigs which had been infected with homologous virus and then hyper-immunized by a second inoculation 12 weeks later. Blood was collected 10 days after the second inoculation. Most of the antibody in hyperimmune serum belongs to the IgG class, which was separated from any IgM by filtering through DEAE-cellulose in 0.01 M-phosphate (pH 7.6). Under these conditions, only IgG molecules pass unadsorbed through the ion-exchanger (Brown, 1960; Almeida et al. 1967).

Antibody of the IgM class was prepared from serum collected 7 days after infection of guinea pigs with the virus. At this stage of the infection most of the antibody activity is present in the IgM fraction. Any IgG present in the serum was separated from IgM by centrifuging the serum in 25 ml. of a 5 to 25% sucrose gradient for 18 hr at 25,000 rev./min., using the SW 25-1 rotor of a Spinco Model L centrifuge. The IgM was present in the bottom 10 ml. of the tube, well separated from IgG (Almeida et al. 1967).

Absorbed IgG preparations were made by mixing excess of the virus fraction (i.e. 12s protein sub-unit or trypsin-treated virus) with hyperimmune serum and incubating at room temperature for 1 hr. The mixture was then diluted to a concentration of 1/10 serum with 0.01 M-phosphate (pH 7.6) and filtered through DEAE-cellulose equilibrated with the same buffer solution. The IgG passed through the column unadsorbed. Under these conditions the virus fractions are retained on the ion-exchanger.

The virus-neutralizing activity of the sera and antibody fractions was measured by inoculating 7-day-old mice with 0.03 ml. of mixtures containing equal volumes of serial 10-fold dilutions of virus and the antisera or antibody preparations. The difference between the titration end points of virus plus normal serum and the experimental mixture was taken as the neutralizing activity of 0.015 ml. of the preparation.

**Agar immunodiffusion.** The double diffusion method described by Ouchterlony (1949) was used with minor modifications. Agar (Bacto-Difco, 1%) in tris-HCl buffer (pH 7.6) containing 1/10,000 sodium azide was used and the reactions were observed for 7 days.
Fig. 1. Electron micrographs of foot-and-mouth disease virus: (a) alone; (b) complexed with IgM; (c) complexed with IgG.
Electron microscopy. Purified antibody fractions were used for the complexing experiments. These were mixed with purified virus and stored at 4° for 18 hr. The complexes were then collected by centrifuging at 30,000 rev./min. for 1 hr in the SW39 rotor of a Spinco Model L centrifuge. After removing the supernatant fluid and draining well, the pellets were

Fig. 2. Precipitin lines formed when a mixture of intact virus (V), the 12s protein sub-unit of the virus and each component above are diffused towards hyperimmune (IgG) and 7 days post-infection (IgM) sera. The intact virus produces a line with IgG and IgM but the 12s sub-unit produces a line only with IgG.

Fig. 3. Precipitin lines formed when a mixture of the virus (V) and 12s components, 12s component alone and trypsin-treated virus (T) are diffused towards hyperimmune serum (IgG) or serum which has been absorbed with excess 12s component (12s abs IgG). Note that the trypsin-treated virus gives a line with the absorbed serum. The line fusing with the 12s component produced by the trypsin-treated virus and hyperimmune serum is due to partial breakdown of the preparation to 12s sub-units on storage.
suspended in one drop of distilled water, mixed with an equal volume of 3% phosphotungstic acid adjusted to pH 7.0 with potassium hydroxide, and examined in a Siemens Elmiskop I.

RESULTS

Reaction of virus with IgG and IgM

Virus particles form complexes with each of the antibody classes (Brown, 1960; Almeida et al. 1967). In the electron microscope the IgG complexes were blurred and the outline of the virus particles obscure (Almeida et al. 1967, and Fig. 1c), presumably because antibody was attached to the entire surface of the virus particles. In contrast, the outline of the virus particles in the IgM complexes (Fig. 1b) was as sharp as that of untreated virus particles (Fig. 1a) and attachment of the antibody molecules appeared to be at distinct, regularly spaced sites.

Fig. 4. Electron micrographs of the virus complexed with IgG absorbed with 12s sub-unit.

These observations suggested that the virus possesses more than one type of combining site and support for this was found in agar diffusion experiments. Virus particles, which sediment at 140s, are degraded into the virus RNA and protein sub-units sedimenting at 12s when heated to 56° or when the pH is lowered below 7.0. Whereas the undegraded virus particles gave a precipitin line with hyperimmune serum (IgG) or with 7 days post-infection serum (IgM), the 12s protein sub-unit gave a precipitin line only with IgG (Fig. 2). This result would be expected if the virus possessed two types of combining site, both reacting with IgG but only one reacting with IgM.
Reaction of virus with absorbed IgG

Further evidence that hyperimmune serum contains antibody to two kinds of virus protein was obtained by using IgG which had been absorbed with excess of the 12S protein sub-unit of the virus. Hyperimmune serum was mixed with an amount of the 12S protein sub-unit sufficient to prevent the serum from producing a precipitin line in agar with the 12S protein sub-unit (Fig. 3). The mixture still gave a precipitin line with virus particles and it also gave a reaction with hyperimmune serum, showing that it contained excess of the

12S component. The mixture was diluted with 0.01 M-phosphate (pH 7.6) and passed through DEAE-cellulose in the same buffer solution. The fraction passing through the DEAE-cellulose in the void volume had a virus-neutralizing activity almost as high as that of a filtrate prepared similarly from the same volume of unabsorbed serum (Table 1). The IgG prepared from the absorbed serum formed complexes with virus particles which were similar to those formed with IgM (compare Figs. 1b and 4). The major difference appeared to be that the virus particles in the IgM complexes were further apart, presumably because IgM molecules are longer than IgG molecules (Almeida et al. 1967). The outline of the virus particles in the absorbed IgG complexes was clear in comparison with those in the
Fig. 6. Electron micrographs of the virus complexed with IgG absorbed with trypsin-treated virus particles.
Fig. 7. Electron micrographs of trypsin-treated virus particles (a) alone and (b) complexed with IgG.
Fig. 8. Electron micrographs of trypsin-treated virus particles: (a) with IgM and (b) complexed with IgG absorbed with 12s component. The regularly spaced sites of attachment are clearly shown in the arrowed particles.
unabsorbed IgG complexes and the antibody molecules appeared to be attached at regularly spaced sites rather than to the entire virus surface.

**Evidence for a third combining site on the virus**

Virus which has been incubated with trypsin has several properties which distinguish it clearly from the untreated virus (Wild & Brown, 1967; Wild, Burroughs & Brown, 1969). As far as the present study is concerned, the relevant difference is that trypsin-treated virus, although similar to untreated virus in size and appearance, does not absorb virus-neutralizing antibody from hyperimmune serum (Table 1). Similarly, the absorbed serum gave a precipitin line with the 140S component in agar tests (Fig. 5) but not with the 12S protein sub-unit. The complexes of virus and IgG prepared from serum absorbed with trypsin-treated virus (Fig. 6) were similar to those obtained with 12S-absorbed IgG (Fig. 4) in that attachment appeared to occur only at sites situated at regular intervals on the virus surface. The virus particles in some of the complexes obtained with IgG absorbed with trypsin-treated virus were packed in regular arrays (Figs. 6b and c).

Trypsin-treated virus gave a precipitin line with hyperimmune serum and formed complexes with IgG prepared from the serum (Fig. 7b). The complexes appeared slightly blurred, with the majority of particles closely packed. There was no evidence for antibody attachment at regularly spaced sites. The trypsin-treated virus also gave a precipitin line with hyperimmune serum which had been absorbed with 12S sub-units (Fig. 3) and formed complexes with IgG prepared from the absorbed serum (Fig. 8b). The virus particles in these complexes had a sharp outline and showed distinct, regularly spaced sites of attachment. These observations show that trypsin-treated virus contains at least two types of combining site. Taken in conjunction with the observation that untreated virus reacted with IgG absorbed with trypsin-treated virus, it appears that the virus contains at least three types of combining site.

**Effect of trypsin on reaction of virus with IgM**

Trypsin-treated virus gave only a faint precipitin band with 7 days post-infection serum (Fig. 9) and did not produce any complexes with IgM separated from the serum by sucrose gradient centrifugation (Fig. 8a). These results suggest that IgM does not react with trypsin-
treated virus since the precipitin band obtained with the unfractionated serum could have been produced by traces of IgG. Any IgG would have been removed by the sucrose gradient centrifugation procedure used for preparing the IgM. It seems likely that the IgM is monospecific, reacting only with the combining site removed by trypsin.

**DISCUSSION**

The model shown in Fig. 10 would account for the virus-antibody reactions described in this paper. For convenience, the model is based on an icosahedron because there is no firm evidence to support any other structure. The virus model shown in Fig. 10 possesses three types of combining site situated at the vertices (A and B) and on the faces (C). The regular spacing of the attachment sites observed with the absorbed IgG preparations is best seen in the particles showing a hexagonal profile (compare arrowed particles in Fig. 8 with the profiles of the model shown in Fig. 10). The evidence presented here does not eliminate the possibility that site B is not a surface antigen but is revealed only when site A is removed by trypsin. In a previous report, however, we showed by immunodiffusion experiments that, in addition to the line of identity, it was possible to detect a faint but distinct spur when untreated and trypsin-treated virus preparations were diffused from adjacent wells towards hyperimmune serum (Wild et al. 1969). This result suggests that the trypsin-treated virus has a surface structure identical with the untreated virus except for the antigen (corresponding to the spur) which is removed by the enzyme.

Fig. 11 summarizes the reactions of the virus and trypsin-treated virus with the two classes of antibody. Untreated virus reacts with IgM to give a precipitin line in agar and the complexes are readily visualized in the electron microscope. However, the reaction of the IgM with trypsin-treated virus is much less pronounced. Only a faint precipitin band is
produced when unfractionated 7 days serum is used (Fig. 9) and no complexes could be found in the electron microscope when purified IgM preparations were used for the reaction (Fig. 8a). The 12S protein sub-unit prepared from virus by heating at 56° or lowering the pH below 7.0 does not react with IgM (Brown, 1960, and Fig. 2).

In contrast, the IgG in hyperimmune serum reacts with the virus, either before or after trypsin treatment, and with the 12S protein sub-unit. The complexes which untreated virus particles form with IgG have a blurred appearance (Almeida et al. 1967, and Fig. 1c) and the outline of the virus particles is obscure. By absorbing IgG with either trypsin-treated virus or the 12S protein sub-unit before reacting with virus, the blurred appearance is avoided and the points of attachment of the antibody are then seen at regular intervals on the virus particles (Figs. 4 and 6). In addition, complexes of virus and IgG which has been absorbed with

Fig. 11. Diagram showing the reactions of the virus and trypsin-treated virus with IgM, IgG, IgG absorbed with 12S component and IgG absorbed with trypsin-treated virus.
Electron microscopy of FMDV-combining sites

Trypsin-treated virus are often arrayed in a regular pattern (Fig. 6b) not seen in any of the other complexes. Serum absorbed with trypsin-treated virus contains antibody reacting only with the trypsin-sensitive sites. These sites appear to be situated at the vertices of the virus particles and may allow favourable aggregates to form into ordered patterns. Fig. 12 shows a possible arrangement based on one of the complexes in Fig. 6b.

![Figure 12](image)

Fig. 12. Arrangement of virus particles suggested by the complex shown in Fig. 6b. Only the sites which can be removed by trypsin are involved in the complexing with this absorbed antibody. This may account for the regular pattern seen in these complexes.

Treatment of the virus with trypsin does not prevent formation of a precipitin band with IgG. As with untreated virus, complexes of trypsin-treated virus and IgG do not show any regularly spaced sites of attachment (Fig. 7b). The particles appeared rather blurred and most of them were closely packed. Trypsin-treated virus also reacts with IgG which has been absorbed with 12s protein sub-units, forming complexes with regularly spaced sites of attachment (Fig. 8b). This reaction points to the presence of a third type of combining site distinct from the face and trypsin-sensitive sites. The model we have proposed would also account for some of the observations made by Cowan (1969) in which variants of the virus were shown by agar diffusion analysis to possess more than one antigenic determinant group.

REFERENCES


(Received 17 November 1969)