A Study of the Cross-reacting Antigens on the Intact Foot-and-Mouth Disease Virus and its 12S Subunits with Antisera Against the Structural Proteins

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SUMMARY

Cross-reactions between two strains of foot-and-mouth disease virus (FMDV) belonging to different serotypes (A and O) were studied with intact virus and virus subunits and antisera produced against the isolated structural proteins. Anti-VP1 type O serum showed cross-reactive neutralizing activity, in contrast to the sera raised against intact virus type O, whereas anti-VP1 type A serum only neutralized homologous virus. Anti-VP2, VP3 and VP4 did not show neutralizing activity. In the enzyme-linked immunosorbent assay and radio-immunoassay anti-VP1, -VP2 and -VP3 sera reacted with the 12S virus subunits of both serotypes. No activity was obtained against VP4. Competition experiments with virus subunits of virus type A and O show that anti-VP1 serum is the most type-specific. Anti-VP2 serum is completely cross-reactive, while anti-VP3 serum reacted in an intermediate way. The identical reactions obtained with anti-VP2 sera and the homologous and heterologous virus subunits suggest that the exposed VP2 antigens are identical.

INTRODUCTION

It is generally accepted that intact picornaviruses have the D antigenic conformation. This conformation is responsible for induction of serotype-specific neutralizing activity and cell attachment. Degraded picornaviruses possess the C antigenic conformation, they do not induce neutralizing activity but expose internal antigens responsible for cross-reactions between serotypes (Rowlands et al. 1969; Forsgren, 1972; Lonberg-Holm & Yin, 1973; Rueckert, 1976; Cooper et al. 1978; Smith, 1978; Rweyemamu & Pay, 1979). Picornaviruses have four structural proteins usually identified as VP1, VP2, VP3 and VP4. They may be defined by their relative position on the virus genome, the order from 5' to 3' being: VP4, VP2, VP3, VP1 (reviewed by Sangar, 1979). Intact picornaviruses expose mainly VP1 (Rueckert, 1976), whereas, after degradation, VP3 and VP4 are also exhibited (Carthew & Martin, 1974; Beneke et al. 1977; Lund et al. 1977; Meloen et al. 1979). Because the intact picornaviruses carry serotype-specific antigens and VP1 is the protein mainly exposed on the virion, these type-specific antigens are probably located on VP1. No information is available, however, on the presence of type-specific antigens on the internal proteins. It is also not clear whether cross-reacting antigens are present on all or only on some of the structural proteins. To gain more insight into these problems with FMDV we studied the antigens on intact 140S FMDV particles of type A subtype 10 and type O subtype 1 and on their virus subunits (12S) with antisera prepared against the isolated structural proteins.
METHODS

Viruses. FMDV type A, subtype 10 and type O, subtype 1, strain BFS 1860 were plaque purified four times on BHK cells. The viruses were then passaged once in BHK suspension culture (Barteling, 1974) before being grown to appropriate amounts in the same system. The viruses were partly purified and concentrated as previously described (Meloen, 1976) except that instead of sucrose gradients, linear CsCl gradients (1:30 to 1:50 g/ml) were used. The purified viruses were diluted twofold with 0.1 M-NaCl, 0.05 M-tris-HCl, pH 7.6, precipitated with 2 vol. acetone at \(-20^\circ C\) and stored at \(-20^\circ C\) until used (Harris & Brown, 1977). 12S particles were prepared by acid treatment of purified 140S as described (Meloen et al. 1979).

Antisera. Antisera against the isolated virus structural proteins were prepared as previously described (Meloen et al. 1979). Briefly, structural proteins of O1 virus were separated on a 10% polyacrylamide gel slab using the discontinuous tris-glycine buffer system of Laemmli (1970) in the presence of 0.1% SDS. The proteins of A10 virus were separated on the same gel system but in the presence of 8 M-urea. The stained protein bands were cut out, dried under nitrogen gas and suspended in an appropriate volume of 0.05 M-phosphate buffer, pH 7.4, containing 0.2 M-NaCl and 0.1% SDS. This suspension was left overnight at 50°C and then emulsified with complete or incomplete Freund's adjuvant. Antisera were raised in duplicate in rabbits, using approx. 150 µg of virus protein emulsified in complete Freund's adjuvant, followed by similar inocula of antigens in incomplete Freund's adjuvant given 4 and 8 weeks later. Antisera against complete virus (140S) and 12S antigen were obtained using three doses of acetylene neuraminidase (AEI)-inactivated and acid-degraded 140S particles in doses of 1 and 10 µg respectively.

Micro-neutralization test (MNT). Neutralizing activity was determined in a microtitre system using secondary pig kidney cells (De Leeuw et al. 1979). The sera were tested in twofold dilutions against about 100 TCID\(_{50}\). Endpoints, determined according to Reed & Muench (1938), are expressed as the negative log of the serum dilution endpoint. Sera were considered positive if their titres were \(\geq 0.45\).

Radio-immunoassay (RIA). Essentially the micromethod described by Crowther (1976) was used. \(^{35}\)S-methionine-labelled virus was incubated with serial dilutions of the specific rabbit antiserum in microtitre plates. After addition of goat anti-rabbit gamma-globulin the precipitates were removed by centrifuging and the radioactivity in the supernatants was counted. The endpoint was taken as the negative log of the serum dilution precipitating 70% of the labelled virus. In competition assays, the serum dilution precipitating 30% of labelled antigen was first determined. This quantity of serum was then incubated for 16 h at 4°C with a constant amount of labelled antigen and increasing amounts of the competing homologous or heterologous antigens. Subsequently goat anti-rabbit gamma-globulin was added, the precipitates were removed and the radioactivity in the supernatant was counted. The amount of unlabelled 140S used for competition was determined spectrophotometrically (Bachrach et al. 1964). The concentrations of the 12S preparations were estimated by the Lowry method (Lowry et al. 1951). For a uniform presentation of the results, in Fig. 2 the amounts of competing antigen are expressed as the theoretical amount of antigen (µg) added to 1 ml of undiluted serum.

Enzyme-linked immunosorbent assay (ELISA). The method described by Abu Elzein & Crowther (1978) was used, with the exception that the plates were coated with antigen in a 0.05 M-phosphate buffer, pH 7.4, containing 0.2 M-NaCl instead of a carbonate buffer, pH 9.6. The antisera were serially diluted in the coated plates. After incubation and washing, the plates were incubated with a 1/1000 dilution of goat anti-rabbit peroxidase conjugate. After washing, the colour was developed with 5-amino-salicylic acid as sub-
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strate. The absorbances were read with a Multiscan apparatus (Titertek) at a wave length of 450 nm. In competition assays the serum dilution giving 70% of the maximum absorbance was first determined for every serum. This quantity of serum was then incubated with increasing amounts of homologous or heterologous antigen in the cups. The following steps (washing, incubation with conjugate, etc.) were carried out as described by Abu Elzein & Crowther (1978). For a uniform presentation of the results, in Fig. 3 the amounts of competing antigen are expressed as the theoretical amount of antigen (µg) added to 1 ml of undiluted serum.

RESULTS

Separation of the structural polypeptides

Fig. 1 shows that the structural proteins of virus O1 separated well on SDS–polyacrylamide gel but not on SDS–urea gel. VP₁ is the trypsin-sensitive protein located on the outside of the virion. VP₄ is the fastest moving protein, not always visible due to its poor staining. VP₂ and VP₃ were identified by comparison of the electrophoresis patterns of complete virus and naturally occurring empty particles. The latter contain the much slower migrating VP₀ instead of VP₁ and VP₂ (Rueckert, 1976). The structural proteins of A₁₀ virus separated well on the urea gel. Under these conditions VP₁ migrated ahead of VP₂ and VP₃.

Neutralizing activity

The neutralizing activity of the antisera against different structural proteins of both viruses were determined using the MNT. The results in Table 1(a) show that anti-VP₁ sera have neutralizing activity in contrast to anti-VP₂, VP₃ and VP₄. Anti-140S and anti-12S also show neutralizing activity. Antisera against various A₁₀ antigens showed no activity against O₁ virus. In contrast, anti-VP₁ and anti-12S type O₁ sera showed unexpected cross-reacting neutralizing activity against A₁₀ virus. Because of this, two new anti-VP₁ type O₁ sera were raised. Again they showed cross-reacting neutralizing activity against A₁₀ virus (data not shown).

Activity measured with RIA and ELISA

Activity against 140S particles was measured with the RIA while that against 12S was measured with both the RIA and ELISA. Titres obtained in the ELISA were generally higher than those found in the RIA.

Similar results were obtained with the 140S particles in the RIA as in the MNT (Table 1a and b) with the exception that one antiserum against VP₁ and one against 140S particles of type A₁₀ showed heterologous activity. In the RIA and ELISA against 12S type A₁₀ and O₁ (Table 1c and d) not only anti-VP₁ but also anti-VP₂ and anti-VP₃ serum of type O₁ reacted. The anti-VP₁, anti-VP₂ and anti-VP₃ sera of type A₁₀ did so in the ELISA but hardly at all in the RIA, probably due to the lower sensitivity of the RIA. No reactions were observed with the anti-VP₄ sera.

The differences between homologous and heterologous activity obtained with anti-VP₁ sera and 140S preparations were considerable. Much less pronounced was the difference in activity of these sera with homologous and heterologous 12S preparations, while, in general, anti-VP₂ and -VP₃ sera gave reactions of the same magnitude with 12S preparations of both serotypes (Table 1c and d).
Fig. 1. Polyacrylamide gel (PAGE) patterns of disrupted FMDV, A₁₀ and O₁.

**Competition experiments**

To study the differences in avidity between homologous and heterologous reactions, competition assays were done with all sera against both homologous and heterologous 140S and 12S preparations. The results with 140S particles are shown in Fig. 2. For reasons discussed below, competition involving 140S particles was done only in the RIA. Because the duplicate sera always showed similar patterns, only those with the highest titred serum of each pair are presented. Anti-VP₁, anti-12S and anti-140S sera showed considerable cross-reactions with heterologous antigen preparations; however, the reactions with homologous preparations were much stronger than with the heterologous
Table 1. Serological activity of antisera against isolated virus structural proteins, complete virus (140S) and virus subunits (12S) of FMDV A₁₀ and O₁.*

<table>
<thead>
<tr>
<th>Sera</th>
<th>(a) MNT</th>
<th>(b) RIA with 140S</th>
<th>(c) RIA with 12S</th>
<th>(d) ELISA with 12S</th>
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<tr>
<td></td>
<td>A₁₀ virus</td>
<td>O₁ virus</td>
<td>A₁₀ virus</td>
<td>O₁ virus</td>
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<tr>
<td>A₁₀ sera</td>
<td></td>
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<td></td>
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<tr>
<td>Anti-VP₁</td>
<td>2.0</td>
<td>2.0†</td>
<td>4.1‡</td>
<td>3.9</td>
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<tr>
<td>Anti-VP₂</td>
<td>—</td>
<td>—</td>
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<tr>
<td>Anti-VP₃</td>
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<td>—</td>
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</tr>
<tr>
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<td>2.0</td>
<td>3.5</td>
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</tr>
<tr>
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<td>1.1</td>
<td>3.7</td>
<td>2.7</td>
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<td></td>
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<td>1.4</td>
<td>2.4</td>
<td>2.3</td>
</tr>
<tr>
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</tr>
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<td>Anti-12S</td>
<td>1.2</td>
<td>2.4</td>
<td>2.6</td>
<td>3.9</td>
</tr>
</tbody>
</table>

* The duplicate results shown are obtained from two separate antisera raised against each antigen.

† Endpoint is expressed as the final negative log of the serum dilution; —, indicates less than 0.3.

‡ Log serum dilution precipitating 50% of the labelled antigen; —, less than 1.6.

§ Log serum dilution giving an absorbance of twice the background; —, less than 1.6.
Fig. 2. Results of competition RIA with 140S particles. In this assay the serum dilution of each serum precipitating 30% of the labelled 140S was first determined. This quantity of serum was then incubated with a constant amount of labelled 140S and increasing amounts of the competing homologous (●—●) or heterologous (▲—▲) 140S. Subsequently, goat anti-rabbit gamma-globulin was added, the precipitates were removed by centrifuging and the quantity of radioactivity left in the supernatant counted. For reasons of uniformity the amount of competing antigen is expressed as the theoretical amount in μg added to 1 ml of undiluted serum. (a, c, e) Type A: anti-VP1 (a), anti-140S (c), anti-12S (e); (b, d, f) type O: anti-VP1 (b), anti-140S (d), anti-12S (f).

ones. This difference was most pronounced for anti-VP1 sera. The sera against O1 virus show somewhat more heterologous reactivity than those against A10 virus.

Competition with 12S preparations was done in both ELISA and RIA, the results being virtually the same. Due to the low reactivity of some sera, the RIA could not be applied throughout, in contrast to the ELISA. Therefore, only ELISA results are shown. Because duplicate sera always gave similar results, the results of only one serum are shown.

Fig. 3 shows that anti-VP1 serum to A10 virus was completely type-specific. Anti-VP2 serum reacted with antigens of both homologous and heterologous 12S preparations with the same avidity, while anti-VP3 serum of type A reacted with heterologous 12S but with lower avidity than with homologous 12S preparations. With anti-140S and anti-12S sera, incomplete cross-reactions were observed.

With anti-VP1, VP2 and VP3 sera to virus O1, complete cross-reactions were obtained with a decreasing difference between avidities of homologous and heterologous reactions (Fig. 3) in the order VP1, VP3, VP2. Anti-VP3 reacted almost identically with homologous and heterologous 12S. As with A10 virus, anti-140S and anti-12S sera against O1 show incomplete cross-reactions.

DISCUSSION

The immunogenicities of isolated VP2 and VP3 are less than that of VP1 (Table 1) and might be related to their insolubility (Strohmaier et al. 1978). We therefore always kept all the isolated structural proteins in solutions containing 0.1% SDS. However, this has
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Fig. 3. Results of the competitive ELISA with 12S virus subunits. In this assay the serum dilution of each serum giving 70% of the maximum absorbance was first determined. This quantity of serum was then incubated with increasing amounts of competing homologous (○ - ○) or heterologous (△ - △) 12S. After incubation and washing, goat anti-rabbit peroxidase was added. After further incubation and washing, the colour was developed with the substrate. For reasons of uniformity the amount of competing antigen is expressed as the theoretical amount in μg added to 1 ml of undiluted serum. (a, c, e, g, i) Type A: anti-VP1 (a), anti-VP2 (c), anti-VP3 (e), anti-140S (g) and anti-12S (i); (b, d, f, h, j) type O: anti-VP1 (b), anti-VP2 (d), anti-VP3 (f), anti-140S (h) and anti-12S (j).

the disadvantage that after injection these preparations not only produced antibodies against the specific antigens but also against the SDS–protein complex (our unpublished results; Lampre et al. 1979). These latter antibodies do not interfere in the RIA or ELISA because in these assays SDS is absent.

Fig. 1 shows that the VP1, VP2 and VP3 of type A10 and VP1 of type O1 preparations used for vaccination were probably homogeneous because these proteins are well separated from each other and are easily cut out of the stained gels in contrast to VP2 and VP3 of O1 virus, where cross-contamination could not be avoided. Further evidence for the absence of cross-contamination is the presence of neutralizing activity in anti-VP1.
sera only. Consistent with earlier observations (Meloen et al. 1979), no anti-VP₄ activity was obtained, which perhaps indicates that VP₄ is insoluble even in the presence of SDS.

The cross-reacting neutralizing activity in the anti-VP₁ sera of O₁ virus against A₁₀ virus is rather unexpected. This activity, which is also present in one anti-12S serum, might be due to some kind of contamination of type O₁ virus with type A virus. Contamination of the proteins after they have been eluted from the gels can be ruled out since the heterologous activity is found only in anti-VP₁ and anti-12S serum but not in the others. Contamination of the virus used for the antigenic preparations however remains possible but seems unlikely because (i) the virus used was cloned and received only a few passages in BHK cells; when tested no heterologous complement fixing activity could be found in it (unpublished data); (ii) some of the anti-VP₁ type O sera also showed neutralizing activity with type C virus in contrast to anti-140S O₁ sera (unpublished data), while at the time when these anti-O₁ sera were prepared no work was being done with type C virus in our laboratory; (iii) ten passages of the O₁ virus on BHK cells in the presence of early convalescent antiserum against O₁ did not result in the appearance of virus with heterologous complement-fixing activity (unpublished data); (iv) contaminant virus present in sufficient amounts to induce neutralizing activity in the anti-VP₁ preparations would probably have shown up in the anti-140S sera but this was not observed. It might be argued that, due to the much larger amounts of VP₁ compared with those of 140S used for vaccination, expression of the contaminant only may have occurred in the anti-VP₁ sera. This argument certainly would apply if the immunogenicity of 140S and VP₁ is of the same order; however, VP₁ is much less immunogenic than 140S. Thus it seems unlikely that a contaminant caused these unexpected reactions. Another explanation for the cross-reacting neutralizing activity of the anti-VP₁ type O₁ sera could be that large amounts of VP₁ used as immunogen raise antibodies with a broad specificity spectrum compared with those raised with small amounts of 140S. This possibility cannot be excluded either but also seems unlikely because with the anti-VP₁ sera of A₁₀ virus such a broad spectrum is not observed. Not only the neutralizing activities but also the competition experiments with 12S antigen (Fig. 3) show a one-way relationship between O₁ and A₁₀. The O₁ anti-VP₁ sera are more cross-reactive than the A₁₀ anti-VP₁ sera. Thus antigens responsible for inducing neutralizing antibodies present on complete virus may differ from those expressed by isolated VP₁ and 12S preparations. It would be of interest if this could be further substantiated, especially since efforts are being made to produce VP₁ of FMDV by genetic manipulation for use as a vaccine (Bachrach et al. 1978; Anon, 1979).

The cross-reactions observed in RIA and ELISA with 12S particles of both strains (Table 1) are in agreement with the observations of others with FMDV and picornaviruses in general. The complete virion having the D antigenicity exposes mainly VP₁ with type- and subtype-specific antigens, whereas 12S particles having the C antigenicity expose internal antigens and are more cross-reactive (Rowlands et al. 1969; Forsgren 1972; Carthew & Martin, 1974; Beneke et al. 1977; Lund et al. 1977; Meloen et al. 1979; Rweyemamu & Pay, 1979).

These cross-reactions were studied further using competition experiments with the RIA for 140S particles and both the RIA and ELISA for 12S preparations. The method of choice throughout would have been the ELISA due to its higher sensitivity compared with the RIA. Unfortunately, in our hands, the 140S antigen, especially of type O, reacted to some extent with anti-VP₂ and anti-VP₃ sera when coated on to the wells of the microtitre plates. These weak but definite reactions were not observed in the RIA (Table 1) and perhaps might indicate that the intact virion changes its conformation to some extent when coated on to a surface. This undesired effect could not be prevented by using a carbonate buffer (pH 9.6) for coating, flexible polyvinyl plates nor by fixation of the
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virus with formaldehyde or glutaraldehyde. Because of this we used the RIA when complete virus was involved. When 12S particles were used both the RIA and ELISA were applied. Except for the lower sensitivity of the RIA no qualitative differences were seen between the tests either in the direct or in the competition assay. This suggests that 12S antigens either free in suspension or adsorbed to a surface behave identically.

Competition of 140S particles of both virus strains and anti-VP1, anti-12S and anti-140S sera (Fig. 2) show cross-reactions with very low avidities, in agreement with the type-specific nature of intact virus. The results of the competition of homologous and heterologous 12S preparations with anti-VP1, -VP2 and -VP3 sera of both O1 and A10 (Fig. 3) show a cross-reaction increasing in the order anti-VP1, anti-VP3, anti-VP2. Anti-VP2 serum of A10 gives identical reactions with 12S preparations of both types. This is less pronounced in the case of anti-VP2 type O1 serum, also due to incomplete specificity of anti-VP2 and anti-VP3 sera. Anti-VP2 type O1 sera showed reproducibly slightly more cross-reaction than anti-VP3 sera. This suggests that if VP2 and VP3 had been completely separated from each other the differences between both experiments might have been larger, with anti-VP2 of O1 perhaps giving identical reactions with 12S preparations of both types. These results indicate that antigens of 12S preparations exposed by VP1 are the most type-specific, those of VP2 are identical while those of VP3 are partly identical. This tendency is in accordance with the results reported by Robson et al. (1980) who showed that for two A and one O strain the amino acid sequences of VP1 and VP3 are highly variable, whereas those of VP2 are relatively more conserved. However, the antigenicity of VP2 and to a lesser extent that of VP3 appears to be more conserved between virus types than their amino acid sequences. One way to explain this is that the variable parts of VP2 and VP3 are buried inside the 12S particle. Another might be that the changed amino acids do not affect the antigenicity. If this latter explanation is correct and if the conserved antigenicity is related to a conserved role of the protein it follows that certain amino acid sequences may vary, perhaps in a more or less random way, because they are less relevant for the functional role of the polypeptide. A comparable phenomenon has been observed by the HA glycoproteins of influenza viruses (anon, 1980). Therefore, our approach might be of additional value to those comparing amino acid sequences, because it reveals similarities not observed otherwise.

As was to be expected, large differences occur when anti-140S sera are tested in competition assays with homologous and heterologous 12S preparations (Fig. 3). It is surprising, however, that the anti-12S sera against O1 in particular are not more cross-reactive, because 12S exposes three proteins which, when isolated, are able to raise antibodies that are considerably more cross-reactive than anti-12S antibodies. A tentative explanation might be the immunogenic dominance of the type-specific antigens (mostly on VP1) of 12S particles over the cross-reacting antigens (on VP2 and VP3) which is perhaps related to the observed poorer immunogenicity of VP2 and VP3.

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