Comparative Biochemical and Serological Analysis of Five Isolates of a Single Serotype of Foot-and-Mouth Disease Virus

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SUMMARY

A comparison has been made of some of the biochemical and serological characteristics of five isolates of foot-and-mouth disease virus (FMDV), serotype A. Three of the viruses have been assigned to the same subtype, A22; the other two belong to different subtypes, A5 and A24. RNA competition hybridization and two-dimensional electrophoresis of the oligonucleotides produced by ribonuclease T1 showed that the three A22 viruses formed a group which could be distinguished from the A5 and A24 viruses. However, the three A22 viruses showed some differences by both tests. Analysis of the virus polypeptides by polyacrylamide gel electrophoresis methods also distinguished the A22 viruses as a group distinct from the A5 and A24 viruses, but small differences within the A22 group were observed using electrofocusing techniques. Serological differences were observed between the viruses using complement fixation tests and by competition radioimmunoassay with antisera obtained from guinea pigs infected with these viruses. The greatest similarity occurred between the viruses previously subtyped as A22, with A5 and A24 being distinct from the A22 group and from each other. The relationship of the biochemical and serological data is discussed.

INTRODUCTION

There are seven serotypes of foot-and-mouth disease virus (FMDV). The degree of difference between the types is such that an animal which has recovered from an infection with one serotype is still fully susceptible to the others, although it is immune against re-infection with virus of the same type. However, when the level of immunity is lower than that found in animals early after infection, as can be found in vaccinated animals, antigenic differences between viruses within one serological type may become apparent by the lack of complete protection against a heterologous strain. These antigenic variants have been called subtypes and the criteria for subtype variation have been reviewed by Brooksby (1968), Fontaine et al. (1968) and Pereira (1977).

Antigenic relationships in FMDV are based on differences between homologous and heterologous reactions in serological tests and are expressed as ratios. For each pair of viruses, two ratios (r1 and r2) of heterologous over homologous serum titres are obtained and a reciprocal relationship is calculated either as a product, \( P = r_1 \times r_2 \) of these two ratios (Davie, 1964), or as a percentage \( R = 100\sqrt{r_1 \times r_2} \) (Archetti & Horsfall, 1950; Ubertini et al. 1964). On the basis of such relationships using complement fixation tests, Brooksby (1968) proposed that strains related by 70 % or more should be considered as belonging to the same subtype, by 32 to 70 % as different subtypes, by 10 to 32 % as widely different subtypes and by less than 10 % as different types. While this approach has given a useful baseline for the serological differentiation of FMDV subtypes, strict adherence to...
these criteria may present problems, e.g. in the case of type A₂₂ viruses which although related on epidemiological grounds show antigenic differences of an order which would justify their classification as distinct subtypes (Arrowsmith, 1975). This led to a re-appraisal of the criteria used for allotting viruses to different subtypes and Forman (1974) and Arrowsmith (1975) proposed that viruses related by an R value as low as 25% should be regarded as variants within the same subtype. The important point, however, is that the serological examination should allow us to predict whether the strain to be used in the production of a vaccine will protect in a field outbreak.

The purpose of the present work was to determine whether an examination of the biochemical properties of the viruses would help in determining the extent of the relatedness between viruses belonging to the same subtype. In this study we have compared the properties of isolates assigned to subtype A₂₂ on epidemiological grounds and by using the Forman (1974) and Arrowsmith (1975) criteria, with two viruses belonging to the distinct subtypes A₆ and A₄₄.

METHODS

Viruses. The following isolates adapted to growth in BHK 21 cell monolayer tissue culture were used: A₆/Germany/1948 (Westerwald), A₂₂/Iraq/1964 (24/64), A₂₂/Turkey/1970 (1/70), A₂₂/Greece/1972 (1/72) and A₄₄/Brazil/1955 (Cruzeiro). These were obtained from the World Reference Centre for FMD, at this Institute. The identity of the viruses was not known by the authors until the experiments were completed.

Preparation and purification of radioactively labelled and unlabelled virus. BHK cell monolayers containing approx. 10⁸ cells were infected at 37 °C at a multiplicity of about 20. After 30 min to allow adsorption, the cells were washed with the appropriate labelling medium and then incubated at 37 °C. Radioactive label was added to the infected cells 1 to 1.5 h p.i. For labelling of the RNA 300 μCi of 5-³H-uridine (26 Ci/mmol) in 20 ml of Eagle's medium were used and for labelling of the protein 100 to 150 μCi of ³⁵S-methionine (100 Ci/mmol) in methionine-free Eagle's medium were used. High sp. act. labelling of virus RNA with ³²P was achieved with 500 μCi/ml of carrier-free ³²P-orthophosphate in phosphate-free Earle's saline added at the times indicated above. All isotopes were purchased from the Radiochemical Centre, Amersham, U.K.

Virus was purified at the end of the growth cycle as described previously (Harris & Brown, 1977) except that the sucrose gradients were centrifuged at 100,000 g for 3 h at 10 °C (Beckman rotor SW 27). Unlabelled virus was prepared from the medium of 4 x 10⁸ BHK cells infected at low multiplicity; after concentration with 50% (NH₄)₂SO₄, buffered to pH 7.6 with 0.04 M-phosphate, it was purified as for radioactive virus.

Extraction and purification of RNA from virus particles. The sucrose gradient fractions containing the virus peak were combined and diluted at least threefold with 0.15 M-NaCl, 0.05 M-tris-HCl, 0.005 M-EDTA, pH 7.6, (TNE buffer) containing 0.1% SDS. Escherichia coli tRNA (100 μg) was then added, the solution extracted once with a 1:1 mixture of phenol:chloroform and the RNA in the aqueous phase precipitated with 2 vol. ethanol overnight at −20 °C. The precipitated RNA was dissolved in 0.5 ml 0.1 M-acetate, 0.1% SDS, pH 5.0, layered on to a 14 ml, 5 to 25% (w/v) sucrose gradient in 0.1 M-acetate, 0.1% SDS, pH 5.0, and centrifuged at 50,000 g for 16 h at 20 °C (Beckman SW 27.1 rotor). The fractions comprising the RNA peak (at about 37S) were pooled, 100 μg of carrier E. coli tRNA added and the RNA precipitated with 2 vol. ethanol overnight at −20 °C. Unlabelled virus RNA was extracted and purified in a similar way, but without adding tRNA carrier.

Extraction of double-stranded RNA from infected cells. Double-stranded RNA (dsRNA) was extracted and purified from about 4 x 10⁸ virus infected cells by phenol extraction and 2 M-LiCl precipitation. The dsRNA in the LiCl-soluble fraction was purified further from
low mol. wt. single-stranded RNA (ssRNA) by centrifuging on 14 ml 5 to 25 % (w/v) sucrose gradients in 0·1 M-acetate, 0·1 % SDS, pH 5·0, for 16 h at 80000 g (Beckman 27.1 rotor) at 20 °C. The dsRNA was recovered from the gradient by ethanol precipitation. These procedures have been described in detail previously by Harris & Brown (1977).

Molecular hybridization. Saturation and competition hybridization experiments were done in duplicate in 0·2 ml of 50 % formamide, 5 x SSC (0·75 M-NaCl, 0·075 M-Na-citrate) at 50 °C, exactly as described by Harris & Brown (1977). The competitor RNAs were dissolved in 50 % formamide, 5 x SSC to give a concentration of 8 μg/ml. The dsRNA concentration was adjusted so that at zero competitor concentration there were between 1000 and 2000 ct/min in the hybrid as determined by saturation hybridization (Harris & Brown, 1977). The results obtained with the hybridization procedures were handled as described in detail by Darby & Minson (1973). Briefly, the reciprocal plots of the competition hybridization give intercepts on the 1/f axis which allow for the determination of f, the fraction of RNA displaced by the competitor at infinite competitor RNA concentration. The values given in Table I are the mean values for the 1/f intercepts from two experiments; the regression coefficient r2 for each of these reciprocal plots was ≥ 0·98. The same results were obtained when the graphical procedure of Eisenthal & Cornish-Bowden (1974) was used directly to ascertain the value of f.

Ribonuclease T1 digestion of virus RNA. The RNA precipitates were dissolved in 10 μl of 0·01 M-tris-HCl, 0·01 M-EDTA, pH 7·4, containing ribonuclease T1 at an enzyme:substrate ratio of 1:20 and the solution incubated at 37 °C.

Polyacrylamide gel electrophoresis of oligonucleotides. Two-dimensional polyacrylamide gel electrophoresis was performed using the method of De Wachter & Fiers (1972) modified as described by Frisby et al. (1976). The electrophoresis cells were 35 × 20 cm in the first dimension with loading pockets 1 cm wide and 30 × 35 cm in the second dimension. The bromophenol blue dye marker was run 17·5 cm in the first dimension and 19 cm in the second dimension. The gels were blotted dry, wrapped in thin plastic sheeting and autoradiographed either directly with Kodirex, Cronex or Fuji film or by the technique of Laskey & Mills (1977), in which pre-flashed Fuji film was used in conjunction with intensifying screens; exposure took place at 4 °C.

Polyacrylamide gel electrophoresis of virus structural proteins. Sucrose gradient fractions containing 35S-methionine-labelled virus particles and carrier bovine serum albumin were diluted twofold with 0·1 M-NaCl, 0·05 M-tris-HCl, pH 7·6, and the virus precipitated with 2 vol. acetone at −20 °C overnight. The precipitate, which contained at least 90 % of the virus, was dried and disrupted in 0·125 M-tris-HCl, pH 6·8, 1 % SDS, 10 % glycerol, 0·2 % dithiothreitol in boiling water for 5 min. The virus polypeptides were analysed on 12·5 % and 10 % polyacrylamide slab gels using the discontinuous buffer system of Laemmli (1970) except that the gels also contained 8 M-urea and were run at 40 V overnight. The slab gels were then prepared for autoradiography as described in detail by Harris & Brown (1975) which permitted the location of the structural polypeptides.

Electrofocusing of the virus structural proteins. Electrofocusing procedures were based on O'Farrell's methods (1975), with the following modifications; Nonidet P40 was omitted from the gel, pH 3·5 to 10·0 Ampholine (LKB Uppsala, Sweden) was used for electrofocusing VP1, VP2 and VP3 towards the cathode, and a mixture of 2:3:5 parts of pH 2·5 to 4·0:pH 5 to 7·0:pH 3·5 to 10·0 Ampholine for electrofocusing VP2, VP3 and VP4 towards the anode. Both modes of electrofocusing were non-equilibrium (O'Farrell et al. 1977). 35S-methionine-labelled virus was mixed with an unlabelled reference strain of FMDV (type O, Pacheco) and the polypeptides were prepared for electrofocusing towards the cathode by disrupting in 10 mM-tris-HCl, pH 7·4, 1 mM-EDTA at 60 °C for 2 min. The RNA was digested for 1 h at 37 °C in the presence of 10 μg/ml pancreatic ribonuclease. Urea (Aristar,
B.D.H., Poole, Dorset), mercaptoethanol and Nonidet P40 were added to give final concentrations of 9 M, 2 % and 2 % respectively. For the anodic mode of operation the virus mixture was disrupted in 1 % SDS, 2 % mercaptoethanol, 10 % glycerol and 10 mM-tris, by heating to 100 °C for 1 min. Comparison between labelled polypeptides in different gels was achieved by aligning the stained reference bands prior to autoradiography. Electrofocused virus polypeptides were identified by the criteria of size and trypsin-sensitivity, given in Results. Experimental evidence will be presented elsewhere (A. M. Q. King and J. W. I. Newman, unpublished data).

Analysis of the protease products of the structural polypeptides. The structural polypeptides were compared by limited proteolysis in SDS and analysis of the products by the PAGE method of Cleveland et al. (1977). Staphylococcus aureus V8 protease, obtained from Miles Laboratories, Slough, U.K., was used at 0·5 μg per sample. The individual bands were cut out of the dried down gels and rehydrated in 0·125 M-tris·HCl, pH 6·8, 1 mM-EDTA, 0·1 % SDS, before loading into the pockets of a 3 cm stacking gel. When the phenol red had reached the end of the stacking gel the current was switched off and the apparatus placed at 37 °C for 30 min. The electrophoresis was then continued at room temperature until the phenol red had reached the end of the resolving gel. The gels were fixed in methanol:acetic acid: H₂O (2:1:7) and the digestion pattern detected by fluorography (Bonner & Laskey, 1974).

Preparation of antiserum. Virus which had been passaged once by intradermal inoculation of the hind foot pads of guinea pigs was inoculated by the same route into groups of five guinea pigs. Sera collected 21 days later were pooled and stored at −20 °C until used.

Complement fixation tests. Chequerboard complement fixation tests (CF) relating the FMD virus strains were made in 'Microtiter' plates, as described by Forman (1974). Purified viruses and guinea pig antisera, obtained 21 days after infection, were used in the test and viruses were related by r and R %, values as described in the same paper.

Competition radioimmunoassay (RIA). Viruses were compared using competition radioimmunoassay as described by Crowther (1977). Tests were made in wells 6 × 20 mm drilled in Perspex blocks. The purified 35S-methionine labelled viruses were diluted in PBS containing 0·5 % bovine serum albumin (PBS-B), to give approx. 40000 ct/min/ml.

(a) Titration of homologous antiserum. Twofold dilutions of antiserum were prepared (50 μl/well) in PBS-B containing a final concentration of 0·5 % normal guinea pig serum (PBS-BS). Homologous radioactive virus (50 μl) and 50 μl PBS-B were added to each well and the plates were incubated at 4 °C overnight. Rabbit anti-guinea pig serum (50 μl) was then added to each well and the plates incubated at 37 °C for 1 h followed by 15 h at 4 °C. Plates were centrifuged for 15 min at approx. 1000 g using Microtiter plate holders and the radioactivity in 100 μl of the supernatant from each well was measured by liquid scintillation. Antiserum titration curves for each homologous system were constructed relating the percentage of radioactive virus precipitated to the serum dilutions. The dilution of serum necessary to precipitate 70 % of the radioactive virus was used in the subsequent competition assay.

(b) Competition assay. Unlabelled purified viruses of known absorbance at 259 nm were diluted in a twofold series (50 μl, quadruplicate samples) in PBS-B. To each test series of viruses, 50 μl of one of the homologous antisera diluted in PBS-BS were added. The mixture was incubated at 37 °C for 1 h and at 4 °C overnight. The homologous radioactive virus was then added to each competitor series at the same dilution as used in the titration of the antiserum. After incubation at 37 °C for 1 h and 4 °C overnight, unreacted radioactive virus was measured after the addition of rabbit anti-guinea pig antiserum as already described. Controls were included to determine the radioactive virus and homologous antibody reaction in the absence of unlabelled competitor (0 % competition) and to measure the total radioactive counts added to each (100 % competition). The percentage inhibition of the homologous radioactive virus–antibody reaction was plotted against the log₁₀ of the
RNA and structural proteins of FMDV

Table 1. Sequence homology of the RNAs of five strains of FMDV serotype A

<table>
<thead>
<tr>
<th>Unlabelled ss competitor</th>
<th>A5</th>
<th>A22 Iraq 24/64</th>
<th>A22 Turkey 1/70</th>
<th>A22 Greece 1/72</th>
<th>A24</th>
</tr>
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<tbody>
<tr>
<td>A5</td>
<td>100*</td>
<td>74</td>
<td>76</td>
<td>77</td>
<td>87</td>
</tr>
<tr>
<td>A22 Iraq 24/64</td>
<td>74</td>
<td>100</td>
<td>87</td>
<td>87</td>
<td>76</td>
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<tr>
<td>A22 Turkey 1/70</td>
<td>74</td>
<td>86</td>
<td>100</td>
<td>87</td>
<td>76</td>
</tr>
<tr>
<td>A22 Greece 1/72</td>
<td>74</td>
<td>88</td>
<td>87</td>
<td>100</td>
<td>76</td>
</tr>
<tr>
<td>A24</td>
<td>87</td>
<td>75</td>
<td>76</td>
<td>77</td>
<td>100</td>
</tr>
</tbody>
</table>

* The figures denote the % sequence homology.

absorbance of the competing strains. Lines were drawn through points between 10 and 85% competition using linear regression analysis. For each assay the homologous unlabelled virus competition plot was used as the standard, and the competition plots of the heterologous strains were compared to this. The amount of unlabelled virus needed to block 50% of the radioactive homologous virus from precipitation was used to relate the viruses.

RESULTS

Competition hybridization of the virus RNAs

The results obtained by competition hybridization using labelled virus RNA, dsRNA from infected cells and unlabelled virus RNA as competitor are shown in Table 1. The homologous virus RNAs always gave $I/f$ values of 1.0 and so were 100% homologous as expected. The level of homology between the three A22 viruses was 87%; when these viruses were compared to the A5 and A24 viruses the level of homology was about 10% lower. The A5 virus gave 87% homology with the A24 virus.

Ribonuclease T1 oligonucleotide fingerprints of the RNAs

The fact that the five RNAs could be distinguished by RNA competition hybridization indicated that there were regions of the genome that differed. These differences were investigated further by two-dimensional polyacrylamide gel electrophoresis of the specific T1 oligonucleotides of the purified $^{32}$P-labelled RNAs (De Wachter & Fiers, 1972; Frisby et al. 1976). The fingerprints observed after autoradiography of the gels are shown in Fig. 1(a to e). There are similarities in the oligonucleotides produced from the RNAs of the three A22 viruses (Fig. 1c, d, e). The large oligonucleotides that appear to be shared by A22 Iraq and A22 Turkey are numbered 1 to 3, 5 to 12 and 14; those shared by all three A22 viruses are numbered 1, 2, 5, 7, 10, 12 and 14. In general the pattern of A22 Turkey is more similar to A22 Greece than to A22 Iraq, particularly in the region of the oligonucleotides numbered 5 and 14. The oligonucleotide maps of the RNAs from the A5 and A24 viruses differ from the three A22 viruses, as expected from the hybridization data. There do not appear to be any large oligonucleotides shared by all five viruses. The length of the poly-cytidylic acid [poly(C)] tract varies, being longest in A22 Iraq and shortest in the A21 virus. The poly(C) tracts of A22 Turkey and A22 Greece run in similar positions.

Analysis of the virus polypeptides by PAGE

The polypeptide patterns of the five type A viruses using a 12.5% polyacrylamide slab gel containing 8 M-urea are shown in Fig. 2(a). The three A22 viruses gave similar patterns but these differed from the A5 and A24 patterns. Co-electrophoresis of the polypeptides of the three A22 viruses with the appropriate controls (Fig. 2b) show that the polypeptides migrate to similar positions, thus indicating a similarity in their size.

In the presence of 8 M-urea the order of the three largest structural polypeptides was
Fig. 1. Autoradiographs of two-dimensional polyacrylamide gels of the ribonuclease T1-oligonucleotides of the RNAs of (a) A3 Westerwald; (b) A24 Cruzeiro; (c) A22 Iraq 24/64; (d) A22 Turkey 1/70; (e) A22 Greece 1/72. The origin is at the top left hand corner and the large arrows indicate the direction of the electrophoresis. In (c), (d) and (e) the numbers refer to oligonucleotides which are common to two or more of the viruses. The position of the poly (C) tract is indicated by C; x and b refer to the positions of the xylene cyanol FF and bromophenol blue markers respectively.

reversed. VP1 is defined as being the trypsin sensitive polypeptide in 140S particles (Wild et al. 1969), and VP2 as being derived from VP0, being coded next to VP4. VP2 was routinely identified by its trypsin-sensitivity in 12S particles (A. M. Q. King, unpublished data). The order of separation of the virus polypeptides in the 8 M-urea gels is therefore VP3, VP2,
Fig. 2. (a) Autoradiograph of a 12.5% polyacrylamide slab gel of the 35S-methionine labelled polypeptides of the A22 Iraq 24/64, A5 Westerwald, A22 Greece 1/72, A24 Cruzeiro and A22 Turkey 1/70 viruses. (b) Autoradiograph of a 10% polyacrylamide slab gel of the three A22 viruses electrophoresed alone and together.
Fig. 3. Autoradiograph of electrophoresed 35S-methionine labelled virus polypeptides. Electrophoresing was towards (a) the cathode or (b) the anode. Lane 1 = A22 Iraq; 2 = A5 Westerwald; 3 = A22 Greece; 4 = A24 Cruzeiro; 5 = A22 Turkey.

Table 2. Relationship of the five FMDV isolates in complement fixation tests

<table>
<thead>
<tr>
<th></th>
<th>A5 Westerwald</th>
<th>A22 Iraq 24/64</th>
<th>A22 Turkey 1/70</th>
<th>A22 Greece 1/72</th>
<th>A24 Cruzeiro</th>
</tr>
</thead>
<tbody>
<tr>
<td>A5 Westerwald</td>
<td>100*</td>
<td>12</td>
<td>18</td>
<td>12</td>
<td>25</td>
</tr>
<tr>
<td>A22 Iraq</td>
<td>24/64</td>
<td>100</td>
<td>37</td>
<td>31</td>
<td>18</td>
</tr>
<tr>
<td>A22 Turkey</td>
<td>1/70</td>
<td>100</td>
<td>61</td>
<td>21</td>
<td>25</td>
</tr>
<tr>
<td>A22 Greece</td>
<td>1/72</td>
<td>100</td>
<td>100</td>
<td>25</td>
<td>100</td>
</tr>
<tr>
<td>A24 Cruzeiro</td>
<td></td>
<td></td>
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</tbody>
</table>

* The figures denote the R% derived from the r values (see Introduction).
RNA and structural proteins of FMDV

Fig. 4. Fluorographs of 15 % polyacrylamide slab gels of the protease digestion products of (a) VP1 (b) VP2 (c) VP3 (d) VP4 of the five viruses.

Table 3. Relationship of the five FMDV isolates in radioimmunoassays

<table>
<thead>
<tr>
<th>Competitor</th>
<th>A22 Westerwald</th>
<th>A22 Iraq 24/64</th>
<th>A22 Turkey 1/70</th>
<th>A22 Greece 1/72</th>
<th>A24 Cruzeiro</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2 Westerwald</td>
<td>1.0*</td>
<td>470</td>
<td>∞</td>
<td>∞</td>
<td>380</td>
</tr>
<tr>
<td>A2 Iraq 24/64</td>
<td>∞</td>
<td>1.0</td>
<td>40</td>
<td>70</td>
<td>∞</td>
</tr>
<tr>
<td>A2 Turkey 1/70</td>
<td>∞</td>
<td>105</td>
<td>1.0</td>
<td>20</td>
<td>∞</td>
</tr>
<tr>
<td>A2 Greece 1/72</td>
<td>∞</td>
<td>14</td>
<td>14</td>
<td>1.0</td>
<td>∞</td>
</tr>
<tr>
<td>A24 Cruzeiro</td>
<td>∞</td>
<td>∞</td>
<td>∞</td>
<td>∞</td>
<td>1.0</td>
</tr>
</tbody>
</table>

* The figures denote the amount of virus needed to block 50 % of the virus from precipitation with its homologous antiserum, based on a value of 1.0 for the homologous virus.
VP1 and VP4 from the top. These identifications were carried out to permit the interpretation of the protease mapping work.

**Electrofocusing of the structural polypeptides**

The patterns obtained by electrofocusing the structural polypeptides of the five viruses are shown in Fig. 3. Polypeptide VP4 from each of the viruses banded in the same place. The three major polypeptides, VP1, VP2 and VP3 all migrated as multiple species. Minor components, probably chemical derivatives, are characteristically observed on the low pH side of each principal band, although VP2 showed extensive heterogeneity which must be partly of genetic origin (Fig. 3a). Polypeptide VP1 of the three A$_{22}$ viruses had the same isoelectric point but this differed from those obtained for the same polypeptide of the A$_5$ and A$_{24}$ viruses. A similar relationship was found with VP3. Polypeptide VP2 of A$_{22}$ Greece and A$_{22}$ Turkey had the same isoelectric point (Fig. 3b) but this differed slightly from that obtained for VP2 of the third isolate, A$_{22}$ Iraq. The VP2 patterns of A$_5$ and A$_{24}$ viruses differed from each other and from the three A$_{22}$ viruses. Hence, the only charge-change that distinguishes the polypeptides of any of the three A$_{22}$ viruses from each other is in VP2.

**Partial protease digestion products of the virus structural polypeptides**

The fluorographs of the partial protease digestion products of the structural polypeptides are shown in Fig. 4(a to d). In general, the patterns obtained with the products of VP1, VP2 and VP3 showed that the A$_{22}$ viruses formed a group which differed from the A$_5$ and A$_{24}$ viruses. However, there were some exceptions. Thus VP1 of the A$_{24}$ virus had a pattern similar to that of the VP1 polypeptides of the A$_{22}$ viruses and the VP3 polypeptide of A$_{22}$ Greece differed slightly from those of the VP3 polypeptides of the other two A$_{22}$ viruses. The VP4 pattern for all five viruses was similar, containing two major bands.

**Serological relationships**

**Complement fixation tests**

The $R\%$ values in Table 2 indicate that the strongest antigenic relationships occur between the A$_{22}$ viruses, particularly between A$_{22}$ Turkey and A$_{22}$ Greece. The relationship between the A$_5$ and the three A$_{22}$ viruses was very small. Virus A$_{24}$ also showed weak antigenic relationships with the A$_{22}$ viruses and with the A$_5$ virus.

**Radioimmunoassay**

The results relating the five strains using the 21 days convalescent antisera show that the A$_{22}$ viruses are much more closely related to each other than to the A$_5$ and A$_{24}$ viruses (Table 3). Within the A$_{22}$ group, Turkey and Greece are more closely related to each other than to the Iraq virus. However, the latter virus is more closely related to Greece than to Turkey. The A$_5$ and A$_{24}$ viruses are only distantly related to each other.

**DISCUSSION**

The purpose of this study was to determine whether analysis of the RNA and proteins of FMDV particles would be of value in the characterization of different isolates within a serotype. The viruses examined were of interest for two reasons: (1) the A$_5$, A$_{22}$ and A$_{24}$ sub-types are readily distinguishable by serological tests and (2) A Turkey and A Greece, which are closely related serologically, were placed in the A$_{22}$ sub-type (of which the first member was A Iraq) on epidemiological grounds rather than on convincing serological evidence. The RNA hybridization data showed that the level of homology between the
three sub-types ranged from 74 to 87%, which confirms our earlier work with sub-types of serotypes O and A (Robson et al. 1977). The level of homology between the three A22 viruses (87%) was lower than we expected for members of a sub-type and was similar to that between the A5 and A24 viruses. Nevertheless the values obtained showed clearly that the A22 viruses form a group which is distinguished from the A5 and A24 viruses.

The RNase T1 oligonucleotide maps also provided evidence that the A22 viruses are more closely related to each other than to the A5 and A24 viruses. Furthermore the two A22 viruses that are the most closely related serologically, i.e. A Turkey and A Greece also gave the most similar RNase T1 maps. It must be pointed out, however, that the identity of the spots moving to the same positions on the different maps has not been established by further analysis. The maps of the A5 and A24 virus RNAs were so different that it is difficult to explain the high level of homology obtained between them. However, a similar high level of homology was obtained between virus RNAs of sub-types A5 and A10 (Robson et al. 1977) which also gave different maps.

Comparison of the structural polypeptides confirmed the virus groupings deduced from the oligonucleotide maps. The three A22 viruses were indistinguishable from each other on Laemmli discontinuous gels while the A5 and A24 viruses differed both from one other and from the A22 group. Electrophoresing is sensitive to mutations that alter charge, unlike electrophoretic separations in SDS gels which are considered to be determined mainly by size difference. Nevertheless the two methods demonstrated very similar relationships. Conservation of charge was observed for all four structural polypeptides of A Turkey and A Greece while the earlier A Iraq virus showed a difference only in VP2. Minor differences within the A22 group were also detected after cleavage of the structural polypeptides with Staphylococcus aureus V8 protease.

The serological data confirm previous evidence that the three sub-types can be readily distinguished. The competition RIA amplified differences between sub-types and the only significant competition observed with the A5 and A24 sera was with homologous virus. Within the A22 sub-type both the complement fixation tests and the competition RIA experiments indicated a strong relationship between the Turkey and Greece isolates, with less reaction between these viruses and the 'earlier' Iraq virus.

It can be concluded that the biochemical analyses distinguished between the three sub-types and the results gave useful baselines regarding the extent of the differences to be expected between sub-types of FMDV. In particular the RNase T1 maps showed that viruses of different sub-types could be distinguished from each other. The biochemical analyses also supported the inclusion of the type A viruses from Turkey in 1970 and Greece in 1972 within the A22 sub-type, which had been based on epidemiological rather than serological data. Both the serological and biochemical analyses suggest that there was a drift within the A22 sub-type from 1964 to 1972 with changes in the RNA and structural polypeptides reflecting the antigenic variation.

We wish to thank Dr A. J. Beale for arranging the provision of a Wellcome Studentship for K. J. R. H.; Dr D. V. Sangar for suggesting the use of 8 m-urea in the polyacrylamide gel electrophoresis system used for separating the structural polypeptides; and Dr J. B. Brooksby, Dr T. J. R. Harris and Dr H. G. Pereira for helpful discussions.

REFERENCES


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