Biochemical Analysis of a Virulent and an Avirulent Strain of Foot-and-Mouth Disease Virus

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SUMMARY

A comparison has been made of some of the serological and physicochemical properties of a virulent and an avirulent strain of foot-and-mouth disease virus, serotype SAT\(_1\). The avirulent strain (SAT\(_1\)-82) was derived from the virulent strain (SAT\(_1\)-7) by serial passage in BHK 21 cells. The viruses were indistinguishable in cross-neutralization tests. In immunodiffusion tests a clear spur line was obtained with the SAT\(_1\)-82 antiserum but not with SAT\(_1\)-7 antiserum. The major polypeptides of the two viruses were identical when examined by polyacrylamide gel electrophoresis.

Hybridization and thermal denaturation experiments failed to distinguish between the RNAs but two-dimensional electrophoresis of the oligonucleotides produced by ribonuclease T\(_1\) digestion revealed several differences. Possibly the most significant of these differences was the size of the polycytidylic acid [poly (C)] tract. There were about 170 nucleotides in the poly (C) tract of the SAT\(_1\)-7 RNA compared with around 100 in the SAT\(_1\)-82 RNA. Further evidence for this deletion was provided by the slightly different behaviour of the two RNAs when compared by sucrose gradient centrifugation and polyacrylamide gel electrophoresis.

INTRODUCTION

Serial passage of a virulent virus population in an ‘unnatural’ host is a method commonly used to prepare attenuated viruses for use as vaccines. Little is known, however, about the genetic basis of the differences between virulent and attenuated viruses.

Some years ago, work with poliovirus demonstrated that it was possible to group attenuated and neurovirulent viruses on the basis of certain in vitro biological properties; for example, efficiency of plating under agar overlays at different pH (Vogt, Dulbecco & Wenner, 1957), the fact that they contain sulphated polysaccharides (Agol & Chumakova, 1962) or their reproductive capacity at 39 to 40 °C (Lwoff & Lwoff, 1959). The elution characteristics of poliovirus from different adsorbents have also been used as a marker for neurovirulence (Hodes, Zepp & Aimbender, 1960; Woods & Robbins, 1961; Koza, 1963). In addition, serological differences between virulent and attenuated polioviruses have been detected (e.g. Wecker, 1960; Gerber & Birch, 1965). It is not clear whether any of these differences are directly related to the attenuation or merely coincide with it.

Apart from poliovirus, foot-and-mouth disease virus (FMDV) is the only other picornavirus which has been used on a large scale as a live attenuated vaccine (Skinner, 1959, 1960; Hollom, Knight & Skinner, 1962). In 1969, Mowat, Barr & Bennett described the preparation of a strain of FMDV (SAT\(_1\)) which was attenuated for cattle by serial passage...
and cloning in BHK 21 cells. In an extension of our studies to obtain chemical markers for particular virus-directed functions, we have used the virulent and attenuated viruses described by Mowat et al. (1969) to examine the biochemical basis of the changes which occur on the attenuation of this virus population.

METHODS

Viruses. The history of the viruses was described fully by Mowat et al. (1969). They originated from a sample collected during an outbreak of FMDV, type SAT1, at Haci Pasa in Turkey in 1962. The original sample was passaged once in unweaned mice and three times in bovine tongue epithelium before growth in BHK cells. The 7th passage in BHK cells (SAT1-7) was still highly virulent for cattle whereas the clone selected from the 82nd passage (SAT1-82) was not virulent for cattle. Encephalomyocarditis (EMC) virus was grown in BHK cells from a seed provided by Dr R. R. Rueckert.

Growth characteristics and determination of infectivity. The growth cycles of the two viruses were compared by infecting monolayers of BHK cells at 37 °C at an m.o.i. of approx. 10, washing the cells after an adsorption period of 45 min and removing samples of the supernatant fluid at intervals for titration. The viruses were titrated by inoculating tenfold dilutions i.p. into 7-day-old mice and the 50% end-points calculated by the method of Reed & Muench (1938).

Preparation of antisera. Three types of serum were used for testing antigenic specificity: (a) convalescent serum collected 21 days after infecting guinea pigs by intradermal inoculation of the hind foot pads with purified virus; (b) immune serum prepared by inoculating mixtures of inactivated virus (0.05% acetyleneimine at 37 °C for 6 h) and Al(OH)3 gel subcutaneously into guinea pigs. The serum was collected 21 days later; (c) hyperimmune serum collected 21 days after giving a second inoculum of inactivated virus to immune animals. Sera from each group of animals were pooled and stored at -20 °C.

For the comparison of antigenic potency, purified virus from sucrose gradients was inactivated by incubating at 37 °C for 6 h with 0.5% acetyleneimine. Mixtures of tenfold dilutions of the inactivated virus and Al(OH)3 gel were inoculated subcutaneously into groups of guinea pigs and the blood collected 28 days later. The sera from the animals in each group were examined individually.

Serum neutralization tests. Equal volumes of tenfold dilutions of virus and appropriate dilutions of antisera were inoculated i.p. into 7-day-old mice and the difference in the end points of the mixtures and that of the virus alone were taken as the neutralizing activity of 0.015 ml serum dilution.

Immunodiffusion tests. Ouchterlony's method (1948) was used except that 0.85% agarose in 0.15 M-NaCl containing 0.1% sodium azide was used instead of agar. Purified preparations of the virus particles were precipitated from sucrose gradients with 2 vol. acetone at -20 °C, resuspended in a small vol. of buffer solution and allowed to diffuse towards hyper-immune or convalescent serum. The precipitation pattern was recorded daily for 7 days.

Preparation of radioactively labelled viruses. BHK cell monolayers containing approx. 10⁸ cells were infected at 37 °C with virus at high multiplicity. After 30 min to allow adsorption of virus, the cells were washed with the appropriate labelling medium, 20 ml of fresh labelling medium added and the cells incubated at 37 °C. Radioactive label was added to the SAT1-82 virus infected cells 1.5 h after infection and to the SAT1-7 virus infected cells 4 to 5 h after infection. For ³H-uridine labelling, 100 to 500 μCi of ³H-uridine (25 to 30 Ci/mmol) in 20 ml of Eagle's medium was used and for ³⁵S-methionine labelling, 2 to 5
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μCi/ml of 35S-methionine (100 Ci/mmol) in methionine-free Eagle's medium was used. High specific activity labelling of virus RNA with 32P was done with 250 μCi/ml of carrier-free 32P-orthophosphate in phosphate-free Earles' saline added at the times indicated above. All isotopes were purchased from the Radiochemical Centre, Amersham.

Purification of virus. Virus was purified from medium at the end of the growth cycle essentially as described by Brown & Cartwright (1963) using 1% SDS in place of deoxycholate. The sucrose gradient (15 to 45% sucrose in 0.1 M-NaCl, 0.05 M-tris-HCl, pH 7-6), was centrifuged at 80000 g for 2-2.5 h at 10 °C (MSE rotor 59590). The gradient was collected as 1 ml fractions into bijou bottles containing 25 μg of bovine serum albumin and the fractions containing the labelled virus were pooled. Sucrose gradients of unlabelled virus used for competition hybridization were monitored by measuring the extinction at 260 nm.

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, disrupted and analysed on high resolution 12-5% polyacrylamide gel slabs using the discontinuous buffer system of Laemmli (1970) as described in detail previously (Harris & Brown, 1975).

Extraction of RNA from virus particles. For hybridization and oligonucleotide mapping experiments, the sucrose gradient fractions containing virus were diluted at least threefold with 0.15 M-NaCl, 0.05 M-tris-HCl, 0.005 M-EDTA (TNE buffer) containing 0.1% SDS, 100 μg of E. coli tRNA added, extracted twice with a 1:1 mixture of phenol:chloroform and the RNA precipitated with 2 vol. ethanol overnight at -20 °C. Generally the RNA was re-precipitated once with ethanol before use. An alternative procedure was used to prepare RNA for analysis on sucrose gradients or polyacrylamide gels. Acetone precipitated virus was resuspended in 0.5 ml 0.1 M-acetate, 0.1% SDS, pH 5.0, which disrupts the virus, and the RNA separated from protein by centrifugation (5 to 25% sucrose gradient in 0.1 M-acetate/0.1% SDS, pH 5-0, 65000 g 16 h, 20 °C). The RNA peak (at about 37S) was pooled, 100 μg of carrier E. coli tRNA added, and RNA precipitated with 2 vol. of ethanol overnight at -20 °C for further analysis.

Analysis of virus RNA. Sucrose gradient centrifugation was done on 5 to 25% sucrose gradients as described above. Non-denaturing electrophoresis on 2:2% polyacrylamide 0.5% agarose gels was done using the method of Peacock & Dingman (1968) as described in detail by Harris & Wildy (1975). Formamide-polyacrylamide gel electrophoresis was carried out on 3.6% gels using external electrodes (Pinder, Staynov & Gratzer, 1974a).

Extraction of double-stranded RNA from infected cells. BHK cell monolayers (15 to 20 Roux) were infected with virus and incubated at 37 °C until the cells had left the glass. The cells were then collected by centrifugation, resuspended in isotonic buffer (0.14 M-NaCl, 0.01 M-tris-HCl, 0.0015 M-MgCl2, pH 7-6) at a concentration of 106 cells/ml and emulsified with buffer-saturated phenol at room temperature. The aqueous phase from this extraction, which contained most of the cytoplasmic RNA and little nuclear material, was made 0.5% with respect to SDS, extracted twice with an equal vol. of phenol:chloroform (1:1) and the RNA precipitated with ethanol overnight at -20 °C. The precipitate was resuspended in a small volume of TNE buffer containing 0.1% SDS and high mol. wt. single-stranded (ss) RNA precipitated by the addition of an equal vol. of 4 M-LiCl overnight at 0 °C. The supernatant fluid, which contained 4S RNA and the double-stranded (ds) RNA molecules was then precipitated with 2 vol. of ethanol overnight at -20 °C. This precipitate was dissolved in 0.5 ml of 0.1 M-acetate, 0.5% SDS, pH 5.0 and centrifuged on a 5 to 25%
sucrose gradient in the same buffer (80000 g, 25000 rev/min, MSE 59108 rotor, 16 h). The peak of dsRNA sedimenting at about 20S (determined by extinction at 260 nm and from the position of 3H-uridine-labelled dsRNA from swine vesicular disease virus infected cells sedimented in a parallel gradient) was precipitated by the addition of 2 vol. of ethanol overnight at −20 °C. The precipitates were dissolved in 2 ml of 50 % formamide, 5 × SSC (0·75 M-NaCl, 0·075 M-Na citrate) for the hybridization reactions. The SATe-7 dsRNA preparation was at a concentration of approx. 10 μg/ml and the SATe-82 preparation at a concentration of approx. 15 μg/ml based on the extinction of the sucrose gradient fractions at 260 nm.

**Molecular hybridization.** Both saturation hybridization experiments, where increasing concentrations of dsRNA are added to a constant amount of each labelled ssRNA, and competition experiments, where labelled ssRNA is annealed to homologous dsRNA in the presence of increasing concentrations of competing unlabelled ssRNA, were performed. The procedures were based on those described by Minson & Darby (1973). All experiments were done in duplicate in 50 % deionized formamide, 5 × SSC in a total volume of 0·2 ml. 32P virus RNA (usually 2500 to 5000 cpm/min), dsRNA and in competition experiments unlabelled RNA (at a concentration of 8 μg/ml) were mixed and sealed in freeze drying tubes (FBG-Trident Ltd, London). The samples were heated at 110 to 115 °C in a milled heating block for 10 min and the annealing was allowed to take place at 50 °C for 16 h. The tubes were then placed at 0 °C, 50 μg ribonuclease A and 50 units ribonuclease T1 (Worthington Biochemicals) in 0·5 ml water added and the tubes were incubated for 15 min at 37 °C. The undigested RNA was precipitated by the addition of 100 μg of calf thymus DNA (in 0·1 ml) and ice-cold 10 % TCA (0·8 ml). After 15 min at 0 °C the samples were collected on glass fibre discs (Whatman GF 81), washed successively with 10 ml 5 % TCA and 10 ml of ethanol, and air dried. The amount of radioactivity on the discs was determined by liquid scintillation counting.

Preliminary experiments showed that (a) at least 95 % of the dsRNA in 50 % formamide, 5 × SSC was melted after 10 min at 110 to 115 °C and (b) equilibrium was reached in saturation experiments well before 16 h. The residual ribonuclease resistance of virus ssRNA subjected to the annealing procedure was 1·0 to 1·5 %.

The results obtained with the hybridization procedures were handled as described in detail by Darby & Minson (1973). Briefly, the reciprocal plots for the saturation experiments give an intercept which represents the amount of 32P label entering hybrid at infinite dsRNA concentration. For the competition experiments the reciprocal plots give an intercept which represents the fraction of label which is homologous with competitor RNA.

**Thermal denaturation profiles.** Labelled homologous or heterologous RNA–RNA hybrids were prepared by annealing labelled virus RNAs with denatured complementary strands at 50 °C for 16 h in 0·05 ml of 50 % formamide, 5 × SSC in sealed tubes. The samples were cooled to 0 °C and 0·95 ml of water added to lower the salt concentration. The tubes were then resealed, heated at various temperatures for 8 min, and cooled rapidly in a methanol/ice bath. The samples were then adjusted to 1 × SSC, digested with ribonuclease A (50 μg) and ribonuclease T1 (50 units) for 15 min at 37 °C and the remaining TCA-precipitable radioactivity determined as before.

**Ribonuclease T1 digestion of virus RNA.** The RNA precipitates were dissolved in 10 μl of 0·01 M-tris-HCl, 0·001 M-EDTA, pH 7·4, containing ribonuclease T1 (Sankyo, Japan) at an enzyme:substrate ratio of 1:20 and the solution incubated for 1 h at 37 °C.

**Polyacrylamide gel electrophoresis of oligonucleotides.** Two-dimensional gel electrophoresis was performed using the method of De Wachter & Fiers (1972) modified as described by
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Fig. 1. Growth curves of the virulent and attenuated FMD viruses in BHK cell monolayers at 37 °C: ○——○, SAT1-7; ●——●, SAT1-82.

Frisby et al. (1976). The electrophoresis cells were 35 x 20 cm in the first dimension with loading pockets 1 cm wide and 35 x 30 cm in the second dimension. The bromophenol blue dye marker was run 17.5 cm in the first dimension and a similar distance in the second dimension. One-dimensional gel electrophoresis at pH 8.3 in 6 M-urea was carried out by the method of Porter, Carey & Fellner (1974). The 12.5 % polyacrylamide gel, made up in 0.1 M-tris borate, pH 8.3, 0.0025 M-EDTA and 6 M-urea, measured 0.25 x 35 x 20 cm with loading pockets 1 cm wide.

Analysis of oligonucleotides from one- and two-dimensional gels. The radioactive oligonucleotides were eluted from the gels as described by Porter et al. (1974), digested with ribonuclease A in 0.01 M-tris-HCl, 0.001 M-EDTA at an enzyme:substrate ratio of 1:20 and the products fractionated by high-voltage electrophoresis on DEAE paper (Whatman DE 81) in 0.5 % pyridine, 5 % acetic acid, pH 3.5. The identity of the products was determined from their mobilities relative to markers.
Fig. 2. Immunodiffusion of SAT1-7 and SAT1-82 viruses. The antisera (AS) were prepared in guinea pigs by two inoculations of inactivated purified virus particles.

RESULTS

Virus replication in BHK cells

The growth rates of the virulent (SAT1-7) and attenuated (SAT1-82) viruses were compared in BHK 21 cell monolayers. Using an m.o.i. of approx. 10, the SAT1-82 virus grew more rapidly than the SAT1-7 virus and destroyed the cell monolayer in 8 h. However, the final yields were similar (Fig. 1). This result suggests that the SAT1-82 virus stock, which was prepared by undiluted passage, is not contaminated with defective particles, as these would be expected to reduce the virus yield considerably.

Antigenic properties of the two viruses

One of the phenotypic changes that might distinguish virulent from avirulent virus particles is a modification of antigenicity or immunogenicity. This possibility was tested in three ways:

Immunodiffusion tests

Purified virus particles, isolated from sucrose gradients by precipitation with 2 vol. of acetone and resuspension in a small vol. of 0.04 M-phosphate buffer, were allowed to diffuse towards either convalescent serum taken 21 days after infecting guinea pigs or immune serum obtained from guinea pigs which had received one or two inoculations of inactivated purified virus particles. In each experiment a line of identity was obtained but in addition a spur line between the SAT1-82 virus and the homologous antiserum was obtained indicating that SAT1-82 has an antigenic determinant not present on the SAT1-7 virus particles (Fig. 2).

Neutralization tests

Although the presence of an additional antigenic determinant on SAT1-82 virus might have been expected to reduce its neutralization by SAT1-7 antiserum, cross-neutralization tests with convalescent and immune sera did not detect any difference between the two viruses (Table 1).
Table 1. Cross-neutralization tests with SAT\textsubscript{1-7} and SAT\textsubscript{1-82} viruses

<table>
<thead>
<tr>
<th>Serum</th>
<th>Log virus neutralized by 0.015 ml serum</th>
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<tr>
<td></td>
<td>SAT\textsubscript{1-7}</td>
</tr>
<tr>
<td>SAT\textsubscript{1-7} convalescent*</td>
<td>1/10</td>
</tr>
<tr>
<td></td>
<td>1/100</td>
</tr>
<tr>
<td></td>
<td>1/1000</td>
</tr>
<tr>
<td>SAT\textsubscript{1-7} immune†</td>
<td>1/10</td>
</tr>
<tr>
<td></td>
<td>1/100</td>
</tr>
<tr>
<td>SAT\textsubscript{1-82} convalescent*</td>
<td>1/10</td>
</tr>
<tr>
<td></td>
<td>1/100</td>
</tr>
<tr>
<td></td>
<td>1/1000</td>
</tr>
<tr>
<td>SAT\textsubscript{1-82} immune †</td>
<td>1/10</td>
</tr>
<tr>
<td></td>
<td>1/100</td>
</tr>
</tbody>
</table>

* Pooled sera obtained from guinea pigs 22 days after infection.
† Pooled sera obtained from guinea pigs 21 days after inoculation of inactivated virus particles.

Table 2. Vaccination tests with SAT\textsubscript{1-7} and SAT\textsubscript{1-82} viruses

<table>
<thead>
<tr>
<th>Weight of antigen (μg) inoculated into each animal</th>
<th>Log virus neutralized by 0.015 ml serum*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SAT\textsubscript{1-7}</td>
</tr>
<tr>
<td>SAT\textsubscript{1-7} (16)</td>
<td>3.4 (3.1-3.6)</td>
</tr>
<tr>
<td>SAT\textsubscript{1-7} (1)</td>
<td>0.9 (0.8-1.0)</td>
</tr>
<tr>
<td>SAT\textsubscript{1-82} (19)</td>
<td>3.7 (3.2-4.0)</td>
</tr>
<tr>
<td>SAT\textsubscript{1-82} (19)</td>
<td>1.7 (1.2-2.6)</td>
</tr>
</tbody>
</table>

* Individual sera from each of 4 guinea pigs were tested. Each value is the mean of the titres obtained and the figures in parentheses indicate the range of titres.

Vaccination tests

Despite the observation in Table 1 that the sera from infected and vaccinated animals reduced the infectivity of each virus to the same extent, it is still possible that the two viruses are not equally immunogenic, i.e. do not produce the same amount of antibody per unit weight of virus inoculated. This was tested by inoculating serial dilutions of each inactivated virus into groups of guinea pigs and measuring the serum antibody response after 28 days. The results in Table 2 show that there was no detectable difference in the immunogenicity of the two viruses.
Fig. 3. Autoradiograph of a 12.5% polyacrylamide slab gel of SAT1-7 and SAT1-82 virus polypeptides labelled with $^{35}\text{S}\text{-methionine.}$
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Analysis of virus polypeptides

Our studies with various isolates of swine vesicular disease virus, another picornavirus, have demonstrated that viruses which are antigenically distinct in immunodiffusion tests tend to have different polypeptide patterns when analysed by slab gel electrophoresis using a discontinuous buffer system (Harris & Brown, 1975). Moreover, Young & Moon (1975) have claimed recently that virus particles of the vaccine strain of polio type I contain a structural protein which is labile in the presence of SDS, with the result that this polypeptide is absent when the virus particles are examined by SDS gel electrophoresis. This result has been confirmed in our laboratory (T. J. R. Harris, unpublished data). The major structural polypeptides of SAT\(_{7}\) and SAT\(_{82}\), however, appear identical when analysed by slab gel electrophoresis (Fig. 3), although the high mol. wt. minor polypeptides found in highly purified preparations of FMDV (Sangar et al. 1976) are more apparent in this preparation of SAT\(_{7}\) virus than in the SAT\(_{82}\) virus. Fig. 3 indicates, therefore, that the small antigenic difference detected between these two viruses is not reflected in the pattern of the major polypeptides.
Quantitative estimates of the genetic relatedness of a number of picornaviruses have been obtained by RNA–RNA hybridization reactions. These comparative studies have been made either by annealing virus ssRNA to denatured dsRNA extracted from radioactively labelled infected cells (Young, Hoyer & Martin, 1968; Young, 1973; Yin, Lonberg-Holm Chan, 1973; Brown & Wild, 1974; Brown et al. 1976) or by annealing non-radioactive dsRNA to labelled virus ssRNA (Dietzschold et al. 1971; Dietzschold, Kaaden & Ahl, 1972). The latter method, using ssRNA labelled to high specific activity, has the advantage that both saturation and competition experiments can be done and by plotting the results as reciprocals (see Darby & Minson, 1973 and Methods), a more precise estimate of the homology can be obtained. Increasing concentrations of dsRNA from SAT₁-7 and SAT₁-8₂ infected cells (see Methods) were therefore annealed to a constant amount of each ssRNA labelled to high specific activity with ³²P, and the percentage of the radioactivity forming a hybrid was calculated (Fig. 4). Reciprocal plots of the results gave intercepts of 1·20 for both homologous and heterologous reactions. This value indicates that about 83 % of each ssRNA is annealed at infinite dsRNA concentration. The theoretical maximum of 100 % was not obtained, presumably owing to the presence in the dsRNA preparation of small amounts of contaminating unlabelled ssRNA. The different slopes of the lines in Fig. 4(b) were obtained because of differences in the concentration of dsRNA in each preparation (see Methods).

Further evidence of the homology between the SAT₁-7 and SAT₁-8₂ RNA was furnished by the competition experiments shown in Fig. 4(c, d). The reciprocal data in Fig. 4(d) shows an intercept of 1·0 for all four competition reactions, indicating that each labelled ssRNA is prevented from annealing to dsRNA with equal efficiency by either homologous or heterologous competing ssRNA.
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Fig. 6. Autoradiographs of two-dimensional polyacrylamide gels of the ribonuclease T₁-oligo-
nucleotides of (a) SAT₁-7 and (b) SAT₁-8₂ RNAs. Enlarged tracings of the top half of the auto-
radiographs are shown in (c) and (d). The numbers refer to those oligonucleotides which were
analysed by ribonuclease A digestion (Table 4). The shaded spots in (c) are those oligonucleotides
which are absent in SAT₁-8₂ RNA and the arrows in (d) point to those oligonucleotides which
have changed position in SAT₁-8₂ RNA digests compared to SAT₁-7. The origin (O) is at the top
left-hand corner and the large arrows indicate the direction of electrophoresis.

Temperature melting curves of the RNA hybrids

The hybridization experiments in the preceding section would probably not detect small
deletion or point mutations in either the SAT₁-7 or SAT₁-8₂ RNA which might result in
some degree of mismatching of nucleotides on hybridization. To examine the possibility
that heterologous hybrids were less well matched than homologous hybrids, ³²P-labelled
SAT₁-7 and SAT₁-8₂ ssRNAs were annealed to both types of denatured dsRNA and the
resulting hybrids subjected to thermal denaturation. No mismatch could be detected
(Fig. 5). Both homologous and heterologous duplexes had a Tₘ under these conditions of
90 to 90.5 °C, reflecting the relatively high G plus C content of FMDV RNA (Newman,
Table 3. Differences between SAT<sub>1</sub>-7 and SAT<sub>1</sub>-82 oligonucleotide fingerprints

<table>
<thead>
<tr>
<th>Difference</th>
<th>Probable reason</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spot 3 from SAT&lt;sub&gt;1&lt;/sub&gt;-82 RNA moves more slowly in second dimension than spot 3 from SAT&lt;sub&gt;1&lt;/sub&gt;-7 RNA</td>
<td>Gain of one or two nucleotides</td>
</tr>
<tr>
<td>Spot 15 from SAT&lt;sub&gt;1&lt;/sub&gt;-82 RNA moves faster in first dimension than spot 14 from SAT&lt;sub&gt;1&lt;/sub&gt;-7 RNA</td>
<td>Increase in uridine content</td>
</tr>
<tr>
<td>Spot 18 absent from SAT&lt;sub&gt;1&lt;/sub&gt;-82 RNA</td>
<td>Possible deletion</td>
</tr>
<tr>
<td>Spot 19 absent from SAT&lt;sub&gt;1&lt;/sub&gt;-82 RNA</td>
<td>Possible deletion</td>
</tr>
<tr>
<td>Poly (C) from SAT&lt;sub&gt;1&lt;/sub&gt;-82 RNA moves faster in both dimensions than poly (C) from SAT&lt;sub&gt;1&lt;/sub&gt;-7 RNA</td>
<td>Deletion of about 70 nucleotides</td>
</tr>
</tbody>
</table>

* See Fig. 6(c, d).
† It is also possible to explain this difference by the substitution of a nucleotide with a guanyclic acid. This would result in two small products on ribonuclease T<sub>1</sub> digestion which would not be resolved by this gel analysis.
‡ See Fig. 7 and Table 5.

Ribonuclease T<sub>1</sub> oligonucleotide fingerprints of the RNAs

The fact that the SAT<sub>1</sub>-7 and SAT<sub>1</sub>-82 virus RNAs were indistinguishable by the annealing experiments and the temperature melting curves indicates that extensive if not complete sequence homology exists between them. It is not known precisely for RNA–RNA duplexes, however, how much mismatch of nucleotides can occur before the T<sub>m</sub> is affected; for DNA–DNA hybrids a mismatch of 1 % lowers the T<sub>m</sub> by 1 °C. The possibility that there are some small differences in the nucleotide sequences of SAT<sub>1</sub>-7 and SAT<sub>1</sub>-82 RNA not detected by the melting experiments was examined by analysing, by two-dimensional polyacrylamide gel electrophoresis (De Wachter & Fiers, 1972; Frisby et al. 1976), the specific oligonucleotides produced by complete ribonuclease T<sub>1</sub> digestion of the ³²P-labelled RNAs, extracted from purified virus with phenol-chloroform. The fingerprints prepared by autoradiography of the gels are shown in Fig. 6(a, b) together with a key (Fig. 6c, d) of the top halves of the autoradiographs for ease of comparison. The same pattern for each RNA has been obtained four times with four different preparations of each virus. The size heterogeneity of the RNA extracted from virus with phenol-chloroform did not seem to affect the patterns, since the fingerprint from 37S, full length RNA, prepared by sucrose gradient centrifugation was indistinguishable from that of RNA extracted from virus particles. Although the oligonucleotides produced from the two virus RNAs are very similar, some obvious differences are apparent. These are marked in Fig. 6(c, d) and are summarized in Table 3. The composition of the larger oligonucleotides numbered in Fig. 6(c, d) was determined, after elution from the gel, by ribonuclease A digestion and separation of the products by high voltage electrophoresis on DEAE paper at pH 3·5 (Table 4). These analyses confirmed that oligonucleotides moving to the same relative positions in each fingerprint had the same composition. Oligonucleotides which were seen to have changed position in SAT<sub>1</sub>-82 RNA compared to SAT<sub>1</sub>-7 RNA (spot 3 and spot 15 in Fig. 6d and Table 3) gave similar but not identical ribonuclease A products (Table 4). It was not possible from these analyses to be certain of the exact nucleotide substitution responsible for the change in position. From the position in the gels, however, it is likely that spot 3 in SAT<sub>1</sub>-82 is one or two nucleotides longer than the corresponding spot in SAT<sub>1</sub>-7 RNA, and that spot 15 in SAT<sub>1</sub>-82 contains more uridine
Table 4. Characterization of large ribonuclease $T_1$ oligonucleotides from SAT$_{1-7}$ and SAT$_{1-82}$ RNAs

<table>
<thead>
<tr>
<th>Virus RNA</th>
<th>Oligonucleotide spot</th>
<th>Ribonuclease A products*</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAT$_{1-7}$</td>
<td>1 (poly C)</td>
<td>Mostly C, minor amounts AAC, AC</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Not analysed; from previous work it was shown to be the heterogeneous poly A streak present in picornavirus RNAs (Frisby et al. 1976)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1-2 AAC, 1 AU, 1-2 AC, 2-3 C, 1-2 U, G</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1 AAU, 1 AAC, 1 AU, 1 AC, 2 C, 1-2 U, G</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1 AAC, 1-2 AU, 1-2 AC, 3 C, ~ 4 U, G</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>1 AAC, 1 AG, 2 AC, 3 C, 2-3 U</td>
</tr>
<tr>
<td></td>
<td>7†</td>
<td>1 AAAC, 1 AAU, 2 AAC, 1 AG, 1-2 AU, 1-2 AC, 4-5 C, &gt; 4 U, G</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>1 AAAAX (U?), 1 AU, 2 AC, 3-4 C, &gt; 3 U, G</td>
</tr>
<tr>
<td></td>
<td>9‡</td>
<td>2 AC, 1-2 C, multiple U, G</td>
</tr>
<tr>
<td></td>
<td>10‡</td>
<td>1 AU, 1 AC, 2-3 C, &gt; 5 U, G</td>
</tr>
<tr>
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<td>11</td>
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<tr>
<td></td>
<td>12‡</td>
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<tr>
<td></td>
<td>13‡</td>
<td>1 AAG, 1-2 AU, 2-3 AC, 3-4 C, ~ 2U</td>
</tr>
<tr>
<td></td>
<td>14‡</td>
<td>1 AAC, 2 AC, 3-4 C, ~ 4 U, G</td>
</tr>
<tr>
<td></td>
<td>15‡</td>
<td>1 AAACC, 1 AG, 1 AU, 1 AC, 2-3 C, ~ 4U</td>
</tr>
<tr>
<td></td>
<td>16‡</td>
<td>1 AU, 4 C, ~ 5 U, G</td>
</tr>
<tr>
<td></td>
<td>17‡</td>
<td>2-3 C, multiple U, G</td>
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<tr>
<td></td>
<td>18</td>
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</tr>
<tr>
<td></td>
<td>19‡</td>
<td>1 AAU, 2 AC, 4 C, ~ 4 U, G</td>
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<tr>
<td>SAT$_{1-82}$</td>
<td>1 (poly C)</td>
<td>Mostly C, minor amount of AC</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Not analysed, as SAT$_{1-7}$ Spot 2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2 AAC, 1 AU, 1-2 AC, 1-2 C, &gt; 3 U, G</td>
</tr>
<tr>
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<td>4</td>
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</tr>
<tr>
<td></td>
<td>5</td>
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<tr>
<td></td>
<td>6</td>
<td>1 AAC, 1 AG, 2-3 AC, 3 C, 2-3 U</td>
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<tr>
<td></td>
<td>7†</td>
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</tr>
<tr>
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<td>8</td>
<td>1 AAAAX (U?), 1-2 AU, 2 AC, 4 C, ~ 3 U, G</td>
</tr>
<tr>
<td></td>
<td>9‡</td>
<td>2 AC, 2 C, multiple U, G</td>
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<tr>
<td></td>
<td>10‡</td>
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<tr>
<td></td>
<td>11</td>
<td>1 AAAC, 1 AAC, 1 AG, 2-3 AC, 1-2 C, no U</td>
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<tr>
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<td>12</td>
<td>1 AAAAAX (C or U), 2-3 AC, 3 C, 2 U, G</td>
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<td>13</td>
<td>1 AAG, 1 AU, 2 AC, 3 C, 1-2 U</td>
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<td>14‡</td>
<td>1 AAACC, 1 AG, 1 AU, 1 AC, 2-3 C, 4U</td>
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<td>15‡</td>
<td>1 AAC, 1-2 AU, 2-3 AC, 2 C, 4U, G</td>
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<tr>
<td></td>
<td>16‡</td>
<td>1 AU, multiple C, multiple U, G</td>
</tr>
<tr>
<td></td>
<td>17‡</td>
<td>3-4 C, multiple U, G</td>
</tr>
</tbody>
</table>

* The molarity of the products was estimated visually from the autoradiographs.
† Probably a mixture of two oligonucleotides.
‡ In these analyses Up was run off the end of the paper. The number of Up residues was estimated, therefore, from the position of the oligonucleotide in the gel (see Frisby et al. 1976).

than spot 14 in SAT$_{1-7}$ RNA, as it lies in a higher graticule (Fig. 6 and see Frisby et al. 1976).

The other major difference which is apparent from the fingerprints (Table 3) is that the poly(C) tract in SAT$_{1-7}$ RNA, recognized as such by its position in the gel (Frisby et al. 1976) and by ribonuclease A analysis (Table 4), moved more slowly in both dimensions than the poly (C) tract in SAT$_{1-82}$ RNA, consistent with it being longer in the virulent virus
Fig. 7. Ribonuclease T₁ oligonucleotides of SAT₁-8₂, SAT₁-7 and EMC virus RNAs, analysed by one-dimensional polyacrylamide gel electrophoresis at pH 8·3. The numbers refer to those oligonucleotides analysed further by ribonuclease A digestion. A₁, A₂, A₃ are characteristic EMC virus RNA ribonuclease T₁ oligonucleotides (Porter et al. 1974).
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RNA. This difference in size was confirmed by one-dimensional gel electrophoresis at pH 8.3 of the ribonuclease T1 oligonucleotides of 32P-labelled SAT1-7 and SAT1-82 RNA, where the separation is more dependent on size than charge (De Wachter & Fiers, 1972; Porter et al. 1974; Frisby et al. 1976). The oligonucleotides produced by ribonuclease T1 digestion of 32P-labelled EMC virus RNA were included in a separate track as markers (Fig. 7). The poly (C) tract in SAT1-82 RNA is about the same size as that in EMC virus RNA (approx. 100 nucleotides; Porter et al. 1974) whereas that in SAT1-7 RNA is considerably longer. From its mobility in the gels compared to the poly (C) from EMC virus RNA and the three other characteristic T1 oligonucleotides of this virus RNA, A1 (38 nucleotides), A2 (29) and A3 (27) (Porter et al. 1974), the size of the poly (C) tract in SAT1-7
RNA was estimated to be about 170 nucleotides, which is similar to that found in the RNAs of other FMDV strains (Brown et al. 1974). Ribonuclease A digestion of the tracts confirmed that the majority of their nucleotides were cytidylic acid (Table 5) and further suggested that they were present once each in the molecule and were of the size estimated from visual inspection of the gels (Fig. 7). The sub-molar minor band in SAT1-7 RNA (Fig. 7, track 2, 1A) and the material remaining at the origin in this track were also analysed by ribonuclease A digestion and were found to be predominantly cytidylic acid, indicating some minor heterogeneity in the poly (C) region in this RNA. The major band of poly (C) tract in SAT1-7 RNA, however, is about 70 nucleotides longer than that in SAT1-82 RNA.

Estimation of the size of the virus RNA

Since 50 to 100 nucleotides represent about 1 % of the RNA it seemed possible that such a deletion would be detected by sucrose gradient sedimentation or polyacrylamide gel electrophoresis of the intact virus RNA molecules. Fig. 8(a) shows the distribution of radioactivity when 3H-uridine-labelled SAT1-82 virus RNA and 32P-labelled SAT1-7 virus RNA were centrifuged on parallel 5 to 25 % sucrose gradients in 0.1 M-acetate-0.1 % SDS at pH 5.0. The apparent heterogeneity in size of the virus RNA is characteristic of FMDV-RNA (Brown & Wild, 1966; Chatterjee, Bachrach & Polatnick, 1976) and occurs even in RNA prepared by disruption of virus at pH 5. Owing to this heterogeneity only full length RNA was used for further study. The peaks of radioactivity at 37S indicated by the bar in Fig. 8(a) were pooled, precipitated with ethanol in the presence of 50 μg of E. coli tRNA and analysed further by (i) co-sedimentation in a similar sucrose gradient (Fig. 8b) and (ii) electrophoresis in composite 2-2 % acrylamide-0.5 % agarose gels (Fig. 8c). Fig. 8(b, c) show that SAT1-82 RNA had a slower rate of sedimentation and a higher electrophoretic mobility when compared to SAT1-7 RNA under these non-denaturing conditions. It is apparent also, from the gel electrophoresis profiles (Fig. 8c) that despite selecting RNA molecules sedimenting at 37S, some heterogeneity is still present, particularly in the SAT1-7 RNA preparation. Despite this heterogeneity these results and their reproducibility support the evidence obtained by oligonucleotide analysis that SAT1-7 RNA is approx. 70 nucleotides longer than SAT1-82 RNA. Analysis of the RNAs on a formamide gel, however, under what were assumed to be fully denaturing conditions (Pinder, Staynov & Gratzer, 1974b) failed to reveal any difference between the molecules (Fig. 8d). The reason for this is not clear but possibly suggests some conformational difference between the two RNAs. It is perhaps surprising that SAT1-7 RNA did not appear to be any more heterogeneous when
analysed by this method than it did when analysed by gel electrophoresis under non-denaturing conditions (Fig. 8c).

DISCUSSION

This paper describes some of the differences detectable between a virulent FMD virus strain (SAT1-7) and a derivative (SAT1-82) which had been attenuated for cattle by serial passage and cloning in BHK cells. In agreement with kinetic neutralization tests with attenuated and neurovirulent polioviruses (e.g. McBride, 1959), no difference was found between the virulent SAT1-7 and the attenuated SAT1-82 strains of FMD in neutralization tests either with hyperimmune guinea pig serum prepared by vaccination, or with convalescent serum from infected guinea pigs. A line of partial identity was evident, however, in the immunodiffusion tests with SAT1-82 antiserum. It is not possible to conclude whether this alteration results from a conformational change in the virus particle (Lonberg-Holm & Yin, 1973) or is due to an altered amino acid sequence of the virus particle polypeptides. In this context it should be mentioned that the two viruses co-sedimented in sucrose gradients and could not be differentiated reproducibly in caesium chloride isopycnic gradients, which indicates that the two viruses have similar conformations. Recent examination of neuroviral and attenuated polio virus type I polypeptides by tryptic peptide analysis has demonstrated that several amino acid substitutions have occurred in three of the structural polypeptides of the attenuated strain (Young & Moon, 1975) and preliminary tryptic peptide analyses of SAT1-7 and SAT1-82 polypeptides indicate that there is some minor variation in their amino acid sequences (T. J. R. Harris, unpublished observations).

Young & Moon (1975) found by competition hybridization experiments that the vaccine strain of poliovirus type I shared 95% of its RNA sequence with the neurovirulent parent strain. In a comparative analysis of various serotypes and mutants of FMDV, however, Dietzschold et al. (1972) could not detect any difference between the saturation hybridization behaviour of the RNA from a strain of FMDV (serotype O) partially attenuated for cattle and the RNA of the virus from which it was derived. Our SAT1-7 and SAT1-82 annealing results (Fig. 4) concur with those of Dietzschold et al. (1972) and indicate that the RNAs of SAT1-7 and SAT1-82 differ less in their sequences than the RNAs of the virulent poliovirus and its attenuated derivative. This probably means that the amino acid sequences of the polypeptides of SAT1-7 and SAT1-82 are more conserved than those of the two polioviruses, as is suggested by the polyacrylamide gel results (Fig. 3).

The two-dimensional gels show distinct and reproducible differences between the two RNAs (Fig. 6). Since the characteristic long oligonucleotides produced by ribonuclease T1 digestion of SAT1-7 and SAT1-82 RNA only represent about 5% of the molecule, however, it is probable that there are other nucleotide substitutions or deletions occurring in the RNAs, besides those which are apparent in the two-dimensional gels (Fig. 6); these would not be detected if they fell outside those parts of the molecule with few guanine residues. In view of the Tm data (Fig. 5) it seems likely that these nucleotide substitutions will be few and widely spread and the deletions, if present at all, only very small. In this respect it is interesting that the extra nucleotides of poly (C) in SAT1-82 RNA do not affect the hybridization or the Tm curve of the heterologous hybrids (Fig. 4, 5).

The difference in length of the poly (C) region is the only change in the SAT1-82 RNA molecule which could not arise from SAT1-7 RNA by simple nucleotide substitution; all the other apparent deletions could be due to the substitution of one nucleotide with a guanylic acid (see Table 3). If such a change occurred in the middle of the poly (C) tract of SAT1-7 then two molecules of 75 to 80 nucleotides would be produced on ribonuclease
T₁ digestion and thus the poly (C) tract would be twice molar in SAT₁₋₇₂. Both the two-dimensional (Fig. 6) and the one-dimensional (Fig. 7) gels clearly show that the poly (C) tract is of the same molarity in each virus RNA and this is suggested also from the ribonuclease A analysis of the poly (C) tract taken from the one-dimensional gels (Table 5). The only plausible explanation of the difference, therefore, is that some of the poly (C) originally present in SAT₁₋₇ RNA is deleted as the virus is serially passaged in BHK cells. Sedimentation in sucrose gradients and gel electrophoresis of the virus RNAs also suggests that some deletion of SAT₁₋₇ RNA has occurred on passage.

As the poly (C) tract is shorter in the attenuated virus RNA, it is tempting to suggest that the poly (C) region has a role in defining virulence. However, such a conclusion would be premature in view of the other small deletions and point mutations which may have occurred. Examination of attenuated and virulent clones of virus at a passage level intermediate between SAT₁₋₇ and SAT₁₋₈₂ and also of other pairs of virulent and attenuated viruses should clarify this point. The present results show clearly that there are only minor differences in the RNAs of SAT₁₋₇ and SAT₁₋₈₂ and it should now be possible to ascertain the positions on the RNA where these changes occur. Moreover, the results draw attention to the poly (C) region in FMDV RNA and indicate that the location and role of this conserved homopolymer may be important in the function of the RNA.

We wish to thank Dr P. Fellner (Searle Research Laboratories, High Wycombe) for many helpful discussions and for the use of his high voltage electrophoresis apparatus for the ribonuclease A analyses. We also thank Dr G. N. Mowat for providing the virus strains with their passage history and Mr P. Wallbridge for the photographs.

REFERENCES


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