Genetic Markers Associated with Virulence of Foot-and-Mouth Disease Virus.* (28562)

S. Bengtsson, Z. Dinter and L. Philipson
Institute of Virology, Uppsala University, Uppsala, and State Veterinary Institute, Stockholm, Sweden

Genetic differences between virus strains belonging to the same type of virus have attracted much interest since it has been shown that such differences may be partly correlated to differences in virulence. Polioviruses have been extensively studied and several markers have been developed, as reviewed by Plotkin *et al.*(1). Attenuated strains are as a rule rapidly inactivated by heat, do not multiply at high temperatures or form plaques under acid agar overlay, and their multiplication is inhibited by sulfated polysaccharides; virulent strains show reverse properties.

Although studies on the physical characteristics of some foot-and-mouth disease virus (FMDV) strains have been carried out(2,3), no comparative study has been made on any of the recently developed attenuated strains (4,5,6) and the original virulent strains. This study describes differences in resistance to heat and acid inactivation and the effect of dextran sulphate, a sulfated polysaccharide, on multiplication and plaque formation of virulent and avirulent strains of FMDV in various cell systems.

Materials and methods. Cell cultures. Primary cultures of calf kidney cells were prepared from trypsinized kidneys of newborn calves. Growth medium was Hanks' salt solution with 0.5% lactalbumin hydrolysate (Hanks' + LAH) enriched with 10% calf serum. In some experiments swine kidney cultures prepared as previously described(7) and baby hamster kidney cells (BHK 21) prepared and maintained as described by Mac Pherson and Stoker(8) were used.

Viruses. The following strains were used: Three attenuated strains type A_4 Western Germany, type C Detmold and type O_3 Venezuela, which had been passaged 711, 357 and 718 times respectively in calf kidney cultures, and the original virulent strains from which the attenuated strains had been derived. All strains except the original C strain, which was kindly sent us by Dr. W. Pilz of the FMDV Station of Farbenfabrik Bayer AG, Köln, were generously supplied by Dr. A. Mayr of Bundesforschungsanstalt für Viruskrankheiten der Tiere in Tübingen.

The attenuated strains were received as infected cell culture fluids and the original strains as tongue epithelium from experimentally infected cattle.

Culture fluids from the first passage of the strains in calf kidney cultures in our laboratory stored at –60°C were used as virus material.

Virus assays. Serial 10-fold dilutions were inoculated in 0.1 ml amounts on cell monolayers in plastic petri dishes (Falcon TCD 15 × 60 mm) previously washed twice with PBS A. After adsorption for 30 minutes the plates were overlaid with 4 ml of an overlay containing Earle's solution with 0.22% $NaHCO_3$, 0.05% LAH, 2% calf serum and 0.95 special agar noble (Difco) in final concentrations. The dishes were kept in a humidified atmosphere of approximately 5% CO_2 in air and were stained after 2 days by adding 2 ml overlay containing neutral red in a concentration of 1/20,000. Plaques were read on the following day. In some experiments serial 10-fold dilutions were inoculated in 0.1 ml amounts in each of 5 tube cultures. Infectivity titers were calculated according to Kärber(9) and expressed as log 10 units of TCD_{50}/0.1 ml.

Results. Heat inactivation. The strains were heated by immersing samples in a waterbath at 56°C for 15 minutes. The samples were withdrawn, transferred to an icebath and titrated together with unheated controls. A difference between the virulent and attenuated strains was found, the virulent strains

* This investigation was supported in part by a Public Health Service Research Grant from Inst. of Allergy and Infect. Dis.

TABLE I. Inactivation of Virulent and Attenuated Strains of FMDV at 56°C.

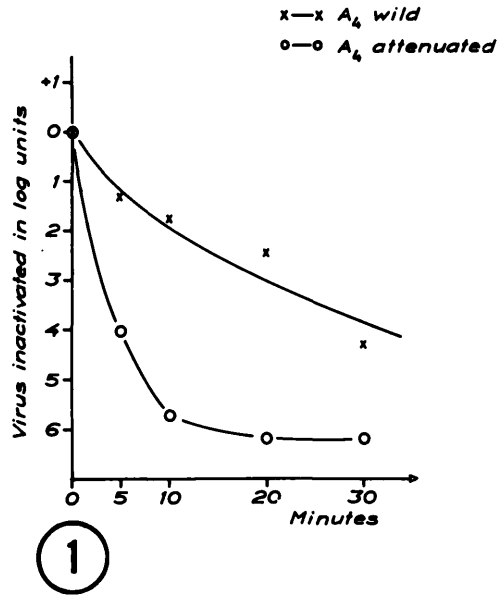
Strain	Titer in log PFU/0.1 ml Control	Heated	Difference	Ratio attenuated/virulent
A_4 virulent	6,6	5,0	1,6	4,3
A_4 attenuated	6,9	1,0	5,9	
C virulent	6,6	4,7	1,9	1,2
C attenuated	6,1	3,0	3,1	
O_3 virulent	6,6	5,0	1,6	>3,6
O_3 attenuated	6,2	<1,0	>5,2	

being more resistant to heat than the attenuated (Table I). It is, however, obvious that this difference is less clear with the C strains than with the other strains. Kinetic studies on rate of heat inactivation were also carried out by heating virus at 56°C and withdrawing samples after various intervals. Fig. 1 shows the rate of inactivation of the two A_4 strains. Similar results were obtained with O_3 strains, whereas the C attenuated strain as expected was less sensitive to heat.

Acid inactivation. The various strains were tested by diluting them 10-fold in 0.1 M citrate-phosphate buffer pH 6.5 and keeping them at room temperature for 10 minutes. Controls were diluted 10-fold in 0.1 M citrate-phosphate buffer pH 7.1. Inactivation was interrupted by diluting the samples 10-fold in 0.2 M tris buffer pH 7.5; the samples were then assayed for infectivity. The results given in Table II show that the attenuated strains are more sensitive to acid inactivation than the virulent, although the difference in sensitivity is less obvious with the O_3 and C strains than with the A_4 strain.

The inactivation of the A_4 strains in citrate-phosphate buffers of different pH is shown in Fig. 2. This confirms that the attenuated strain is more readily inactivated in a slightly acid environment.

Effect of dextran sulphate. The effect of dextran sulphate was tested by inoculating 100 to 1000 TCD_{50} in 0.2 ml onto calf plates washed twice beforehand with PBS A. The virus was adsorbed for 30 minutes at 37°C after which 5 ml of Hanks' + LAH containing 0.01% sodium dextran sulphate 2000 (AB Pharmacia, Uppsala) were added. Controls with ordinary Hanks' + LAH were included. The plates were incubated at 37° and at various times samples were with-



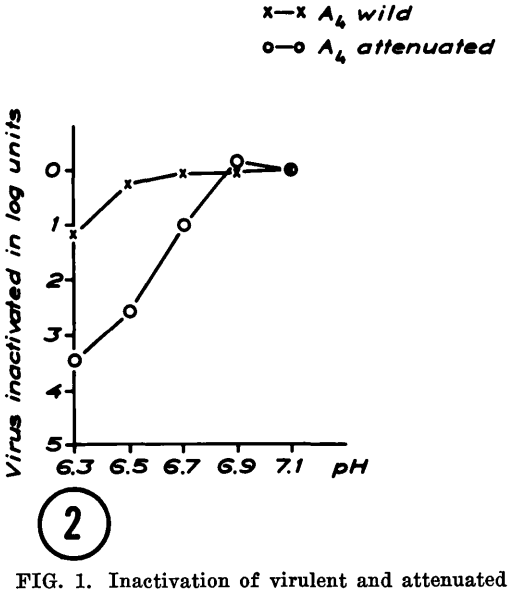


FIG. 1. Inactivation of virulent and attenuated strains of FMDV type A_4 at 56°C.

FIG. 2. Inactivation of virulent and attenuated strains of FMDV type A_4 at various pH.

TABLE II. Inactivation of Virulent and Attenuated Strains of FMDV at pH 6.5.

Strain	Titer in log PFU/0.1 ml pH 7.1	pH 6.5	Difference	Ratio attenuated/virulent
A_4 virulent	7,0	5,6	1,4	>3,7
A_4 attenuated	7,1	<2,0	>5,1	
C virulent	6,0	3,4	2,6	>1,7
C attenuated	6,3	<2,0	>4,3	
O_3 virulent	6,5	4,0	2,5	1,9
O_3 attenuated	6,7	2,3	4,4	

drawn and titrated. The results given in Fig. 3 show that dextran sulphate inhibits multiplication of the attenuated C and O_3 strains. This was confirmed by the finding that the cytopathic effect of these viruses was delayed in cultures containing dextran sulphate in the medium.

Whether dextran sulphate affected the plaque size of the various strains was further tested. The strains were titrated under an agar overlay as described in Methods and also under an overlay containing 0.01% of dextran sulphate 2000. Dextran sulphate diminished the plaque size of the attenuated strains C and O_3 although the decrease in size was less conspicuous for the O_3 than the

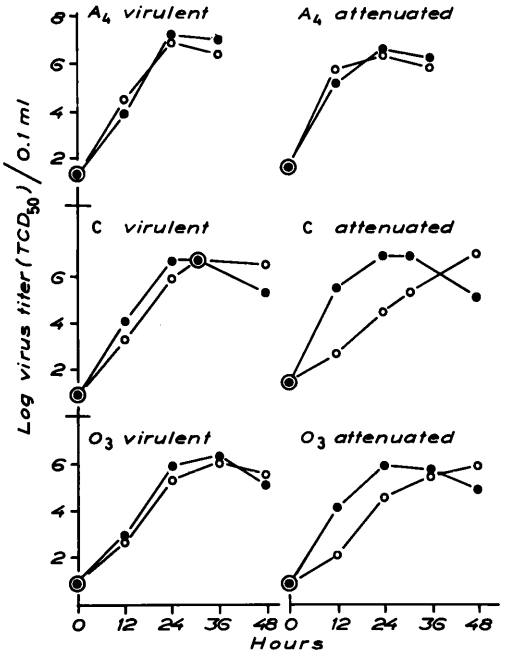


FIG. 3. Multiplication of FMDV strains; ●—●: in Hanks' + LAH, ○—○: in Hanks' + LAH with 0.01% dextran sulphate 2000.

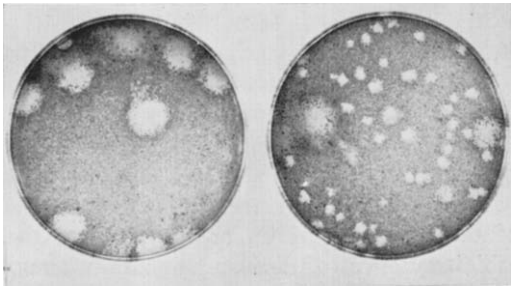


FIG. 4. Plaque morphology of attenuated strain of FMDV type C. Left: with normal overlay; right: with 0.01% dextran sulphate in overlay.

C strain. Fig. 4 shows the appearance of plaques of the C strain in normal plates and in plates with 0.01% dextran sulphate. Some plaques of normal size occur under dextran sulphate. Such plaques and plaques of diminished size were picked and passaged once in calf kidney cultures. They were then titrated in tubes containing Hanks' + LAH or in Hanks' + LAH with 0.01% dextran sulphate. Dextran sulphate has no effect on the titers of the progeny from the large plaques (Table III). These results were con-

TABLE III. Effect of Dextran Sulphate on m— and m+ Mutants of Attenuated Type C Strain of FMDV.

Mutant	Titers in log TCD_{50}/0.1 ml Control	.01 % DS	Difference
m—	6,5	5,0	1,5
m+	6,1	6,0	,1

firmed in plaque tests where the progeny of large plaques gave only large plaques, whereas the progeny of small plaques yielded a mixed population of mainly small with some large plaques, between 5 and 10% of the total.

The effect of dextran sulphate on plaque size of the strains was also tested in swine

kidney cultures and BHK cultures. The results in these cells agreed with the findings in calf cells.

Discussion. It has recently been reported that attenuated and virulent strains of poliovirus type 1 may be quantitatively separated by counter-current distribution in an aqueous polymer phase system containing dextran sulphate and polyethylene glycol(10). The counter-current distribution pattern is correlated to the m-marker(11,12,13), *i.e.*, the sensitivity to sulfated polysaccharides such as dextran sulphate. The aim of this study has been to develop *in vitro* markers for FMDV, to facilitate the application of the counter-current distribution technique to this virus. It has been found that the attenuated strains of the types C and O_3 are sensitive to the inhibitory effect of dextran sulphate, whereas the attenuated A_4 strain was less affected. Mutants sensitive to dextran sulphate or other sulfated polysaccharides, have been found for polioviruses(11,12,13), ECHO 19 (14), Coxsackie B4(15), Dengue(16), FMDV type O(17) and phage T2(18). The mechanism for this inhibition is unknown, but preliminary studies indicate that the penetration of sensitive virus strains is affected(19).

It is known that mutations from sensitivity to resistance occur regularly with poliovirus type 1(10,12); usually between 0.1 to 1% of the plaques are resistant. A similar phenomenon has been observed with the attenuated strain of FMDV type C, although the proportion of resistant plaques is much higher in this case. This may, however, be due to the fallacies of the plaque purification method recently pointed out by Mosley and Enders(20).

Summary. Attenuated strains of FMDV types A_4, C and O_3 are more sensitive to heat and acid inactivation than virulent strains. The attenuated strains of FMDV type C and O_3 are inhibited by dextran sulphate which does not affect the other strains.

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Received June 10, 1963. P.S.E.B.M., 1963, v113.