

Isolation of variants during passage of a strain of foot-and-mouth disease virus in partly immunized cattle

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Subtype antigenic differences exist between strains of the virus of foot-and-mouth disease (FMD) within the seven immunologically distinct types. Reports of effects of this diversity were reviewed briefly in a recent paper (Hyslop, Davie & Carter, 1963). Although it has been suspected that the antigenic changes leading to the appearance of new subtype strains may result from spread of the virus through a partially immune population, direct experimental evidence has been lacking. Recently we have demonstrated subtype changes in strains of FMD virus propagated by serial passage in monolayer cultures of pig kidney cells (PKTC) in which increasing concentrations of strain specific antiserum were incorporated in the medium.

The present report records the serial passage of FMD virus of Type SAT 1 (strain Turkey 323/62) in partially immunized cattle, and the isolation of an immunologically distinct variant after the 34th serial passage.

Virus

MATERIALS AND METHODS

FMD virus of strain Turkey 323/62 (Type SAT 1), originally isolated from a field outbreak near Istanbul, was inoculated into susceptible cattle at Pirbright. A 1/10 suspension (in M/25 phosphate buffer) of infected tongue epithelium from one of these animals, steer DY 90, was clarified by centrifugation and was then inoculated into the lingual mucosa of steer EH 6, a fully susceptible animal. Virus from the resultant vesicles was passaged serially by inoculating clarified suspensions of epithelium into the mucosa of the tongues of partially immunized cattle. In the early passages a 1/10 suspension was employed, but later the dilution was increased to 1/1000, then to 1/10,000 and finally to 1/100,000. Vesicular material was usually harvested from tongue lesions, but on two occasions epithelium was harvested from secondary lesions on the feet, so that only virus capable of generalizing in the host in the presence of circulating antibody was selected on these occasions.

Virus of substrains was propagated also in secondary monolayers of pig kidney cells (PKTC) which were cultured in EYL medium (Earle's saline containing 0.01% yeast extract and 0.5% lactalbumin hydrolysate) for 48 hr. before inoculation of the virus.

Virus titration

Infectivity was titrated by intraperitoneal inoculation of serial 0.5 log dilutions of the virus into five groups of eight unweaned mice of the Pirbright 'P' strain (Skinner, 1951); 50% end-points were estimated by the method of Kärber (1931). The litters were randomized before inoculation.

Cattle

All the cattle were Devon or Devon-cross steers, about 18 months old, which were purchased through a dealer who collected them from various parts of south-west England. During the experiment the animals were housed, usually in pairs, in an isolation compound.

Vaccine

Groups of cattle were vaccinated with one or more graduated doses of inactivated (formalin-treated, aluminium hydroxide adsorbed) vaccine prepared by a modification of the method of Frenkel (1949, 1951, 1953). The vaccine (batch no. 6215), of strain Turkey 323/62, had protected 10/10 cattle against generalized infection when given in the standard 15 ml. dose.

Serum-virus neutralization tests

Cattle were bled immediately before inoculation with virus and their sera were titrated for neutralizing antibody by the tissue culture metabolic inhibition test described by Martin & Chapman (1961), in serial two-fold dilutions against 100 tissue culture ID 50 of virus of each of the substrains isolated during the experiment.

Virus and sera derived from guinea-pigs

Young guinea-pigs, bred in the colony at this Institute, were infected by intradermal inoculation into the left tarsal pad of FMD virus suspensions containing 0.05% (w/v) saponin. Virus of each of the substrains was passaged 2-3 times to adapt it to the guinea-pig. Groups of guinea-pigs were immunized by two doses of virus at 21-day intervals and were exsanguinated 10 days later.

Complement-fixation test (CFT)

Virus from lesions which developed at various stages of the experiment was tested, by the method described by Brooksby (1952), against subtype-specific serum produced in guinea-pigs and also against stock-strain sera of the seven main immunological types supplied by the World Reference Laboratory (WRL). Results were assessed in accordance with the principles recorded by Bradish, Brooksby & Tsubahara (1960) and by Bradish & Brooksby (1960). The cross-fixation ratios (CFR) of the various systems were determined, i.e.

$$\frac{\text{Amount of complement fixed in the heterologous serum-virus system.}}{\text{Amount of complement fixed in the homologous serum-virus system}}$$

Calculation of the product of these ratios (CFP) excluded differences attributable to the relative concentrations of individual reagents, and pairs of strains were considered to be antigenically distinct when their CFP was less than 0.5 (Bradish & Brooksby, 1960).

RESULTS

Clinical results in cattle

Passage of virus in vaccinated cattle

Only one of the vaccinated animals in which the virus was passed (steer EH 7) was without previous experience of FMD virus. Some animals had been used for innocuity tests before being vaccinated and so had received multiple inoculations of 0.1 ml. of inactivated FMD vaccine into the epithelium of the tongue, i.e. 'sensitizing inoculations' (SI), which possibly might have increased their ability to respond to the subsequent vaccination, although none of the animals tested possessed virus-neutralizing antibody titres (SVNT) exceeding 1/6 at the time of vaccination. Other animals had been vaccinated on one or more occasions with inactivated vaccine of strain Turkey 323/62 and possessed considerable levels of strain-specific antibody before the experiment commenced. On the 21st day before the start of serial passage, the animals were grouped on the basis of their known or expected degree of resistance to infection and all were vaccinated subcutaneously with graded doses (0.5–15.0 ml.) of inactivated vaccine. Animals possessing low resistance received smaller doses than those with high resistance and, in the group expected to have the highest resistance, five animals were vaccinated yet again after the start of the experiment. The vaccination history and the immune status of individual animals, as demonstrated by their SVNT immediately before passage of the virus, are shown in Table 1.

The field strain Turkey 323/62 had been inoculated into susceptible cattle 3 times before the experiment commenced. A suspension of infected tongue epithelium (titre $10^{6.9}$ mouse ID 50/ml.) was inoculated into the lingual mucosa of a fully susceptible animal, steer EH 6, and also by the same route into steer ED 89, fully immunized with inactivated vaccine of strain Turkey 323/62. In the susceptible steer, infection generalized from the primary tongue vesicles, part of which was harvested, to produce lesions on the dental pad, gums, muzzle and on all four feet; no lesions developed in the immunized animal. Virus from the lesions of steer EH 6 failed to fix complement with specific sera of types other than Type SAT 1.

The first serial passage in partly immunized cattle was made with a 1/10 suspension of vesicular material from steer EH 6. The recipient animal, steer EH 7, developed a generalized infection but its SVNT was 1/4 and the animal must be considered to have remained fully susceptible at the time of infection. Thereafter, serial passages were made in cattle which possessed detectable antibody against virus of strain Turkey 323/62. The clinical results of infection are summarized for individual animals in Table 1.

Infection generalized in each of the first ten animals; at the 11th passage, generalization ceased but extensive primary lesions continued to occur in every animal. At the 14th passage, secondary lesions appeared again and all succeeding

Table 1. *Passage of FMD virus of strain Turkey 323/62 in partly immunized cattle*

Virus passage no.	Animal no.	History			1/SVNT of sera (pre-infection) against virus of		Virus inoculated		Clinical result of inoculation
		S.I.	Vaccine dose	V-I interval (days)	EC 83 strain 1	EC 83 strain 2	Source	Dilution	
—	EH 6	0	0	—	≤ 3	≤ 3	Tongue DY 90	10 ⁻¹	T D G M 4F
—	ED 89	0	3 × 15 ml.	21	355	≤ 3	Tongue DY 90	10 ⁻¹	Negative
1	EH 7	0	1 × 0.5 ml.	24	4	≤ 3	Tongue EH 6	10 ⁻¹	T D 4F
2	EC 83	1	1 × 0.5 ml.	25	16	≤ 3	Tongue EH 7	10 ⁻¹	T M 4F
3	EC 84	1	1 × 0.5 ml.	26	45	≤ 3	Tongue EC 83	10 ⁻¹	T D 4F
4	EC 87	1	1 × 0.5 ml.	27	90	≤ 3	Tongue EC 84	10 ⁻¹	T 1F
5	EC 89	1	1 × 0.5 ml.	31	256	6	Tongue EC 87	10 ⁻¹	T 2F
6	EC 90	1	1 × 0.5 ml.	32	178	4	Tongue EC 89	10 ⁻¹	T 1F
7	EC 91	1	1 × 1.0 ml.	33	355	16	Tongue EC 90	10 ⁻¹	T D M 2F
8	EC 92	1	1 × 1.0 ml.	34	32	≤ 3	Tongue EC 91	10 ⁻¹	T D 1F
9	EC 93	1	1 × 1.0 ml.	35	178	≤ 3	Tongue EC 92	10 ⁻¹	T 1F
10	ED 50	1	1 × 1.0 ml.	38	45	≤ 3	Tongue EC 93	10 ⁻¹	T D 4F
11	ED 51	1	1 × 1.0 ml.	39	128	4	Tongue ED 50	10 ⁻¹	T
12	ED 52	1	1 × 2.0 ml.	40	45	≤ 3	Tongue ED 51	10 ⁻¹	T
13	ED 53	1	1 × 2.0 ml.	41	90	≤ 3	Tongue ED 52	10 ⁻¹	T
14	ED 54	1	1 × 2.0 ml.	42	22	≤ 3	Tongue ED 53	10 ⁻¹	T 2F
15	ED 55	1	1 × 2.0 ml.	45	32	≤ 3	Tongue ED 54	10 ⁻¹	T 4F
15	ED 56	1	1 × 2.0 ml.	46	32	≤ 3	Tongue ED 55	10 ⁻¹	T 1F
15	EG 84	1	1 × 5.0 ml.	47	N.A.	≤ 3	Tongue ED 56	10 ⁻¹	T 2F
16	EG 85	1	1 × 5.0 ml.	48	45	≤ 3	Foot ED 55	10 ⁻¹	T D 4F
17	EG 86	1	1 × 5.0 ml.	49	90	≤ 3	Tongue EG 85	10 ⁻¹	T D 2F
18	EG 69	1	1 × 5.0 ml.	52	128	≤ 3	Tongue EG 86	10 ⁻¹	T 3F
19	EG 88	1	1 × 5.0 ml.	53	512	16	Tongue EG 69	10 ⁻¹	T 1F
20	EG 89	1	1 × 10 ml.	54	32	≤ 3	Tongue EG 88	10 ⁻¹	T 3F
20	EG 90	1	1 × 10 ml.	59	178	8	Tongue EG 89	10 ⁻¹	T D 1F
21	EG 91	1	1 × 10 ml.	60	N.A.	≤ 3	Foot EG 89	10 ⁻¹	T D 1F
22	EG 92	1	1 × 10 ml.	61	45	4	Tongue EG 91	10 ⁻¹	T 2F
23	EG 93	1	1 × 10 ml.	62	N.A.	≤ 3	Tongue EG 92	10 ⁻¹	T 4F
24	EG 94	1	1 × 15 ml.	63	32	≤ 3	Tongue EG 93	10 ⁻¹	T D 2F
25	EG 95	1	1 × 15 ml.	66	90	≤ 3	Tongue EG 94	10 ⁻¹	T D 4F
26	EG 96	1	1 × 15 ml.	67	32	4	Tongue EG 95	10 ⁻¹	T 3F
27	EG 97	1	1 × 15 ml.	68	32	≤ 3	Tongue EG 96	10 ⁻³	T 3F
28	EG 98	1	1 × 15 ml.	70	32	≤ 3	Tongue EG 97	10 ⁻³	T 1F
28	EG 99	1	2 × 15 ml.	—	N.A.	N.A.	Contact EG 98	—	T 1F
29	EG 68	1	2 × 15 ml.	5	64	4	Tongue EG 98	10 ⁻³	T 1F
30	EH 1	1	2 × 15 ml.	6	178	4	Tongue EG 68	10 ⁻⁴	T 3F
31	ED 85	0	3 × 15 ml.	80	178	6	Tongue EH 1	10 ⁻⁵	T 3F
32	ED 86	0	3 × 15 ml.	81	256	6	Tongue ED 85	10 ⁻⁵	T 1F
33	ED 87	0	4 × 15 ml.	12	256	≤ 3	Tongue ED 86	10 ⁻⁵	T 1F
34	ED 88	0	4 × 15 ml.	13	708	32	Tongue ED 87	10 ⁻⁵	T Died
35a	EH 7	Recovered 77 days after Passage 1			355	11	Tongue ED 88	10 ^{-1.3}	T D 1F
35b	EC 83	Recovered 76 days after Passage 2			512	11	Tongue ED 88	10 ^{-1.3}	T D 2F

- S.I. Sensitizing inoculation (20 × 0.1 ml. vaccine i.d. tongue).
V-I Interval between last vaccination and inoculation of virus.
SVNT Virus neutralizing titre of serum.
T Primary vesicles on tongue.
D Secondary vesicles on dental pad.
G Secondary vesicles on gums.
M Secondary vesicles on muzzle.
1-4F Secondary vesicles on 1 or more feet.

animals showed some degree of generalization, though occasionally (e.g. at the 18th passage) both primary and secondary lesions were delayed.

After the 15th and 20th serial passages, infected epithelium was harvested from secondary lesions on the feet instead of from primary lesions, and filtrates prepared from this material were inoculated into the tongues of the next animals. The 21st and all subsequent passages were made in cattle vaccinated with the normal field dose (15 ml.) on at least one occasion.

The inoculum was diluted 1/1000 after the 26th passage and the dilution was increased to 1/10,000 for the 30th passage, then to 1/100,000 for the 31st and subsequent passages. Although satisfactory primary lesions developed, the secondary lesions were sometimes small and their development was often delayed until somewhat later than might have been expected as a result of the inoculation of virus into fully susceptible cattle.

At the 28th passage, steer EG 98 developed extensive primary lesions and a secondary lesion on one foot. Steer EG 99 was not infected by inoculation but was allowed to remain in contact with steer EG 98 and to drink from the same water bowl. On the 4th day after the appearance of primary lesions on the tongue of steer EG 98, two primary vesicles were observed on the tongue of steer EG 99 and infection generalized later to one foot; CF tests showed that the virus present in these lesions was of Type SAT 1.

The penultimate animal of the series had been vaccinated with 15 ml. doses on four occasions before infection. Nevertheless, three primary lesions and a single secondary lesion resulted. The last animal, steer ED 88, which had been vaccinated similarly 199, 164, 83 and 13 days before infection showed primary lesions at two of the ten inoculation sites despite a SVNT of 1/708 against virus of strain Turkey 323/62. This steer died under general anaesthesia, during harvesting of the primary vesicles, before development of secondary lesions was possible. CF tests employing the harvested material indicated that virus of Type SAT 1 only was present.

Passage of virus in recovered cattle

On the 76th day after primary infection, steer EH 6, the 1st (unvaccinated) animal in the experiment, was reinoculated at ten sites on the tongue with a 1/10 suspension of epithelium from its own original reaction. At this time all lesions had healed completely and the SVNT was 1/1024. No lesions resulted from the second attempt to infect the animal and thus strain Turkey 323/62 produced complete immunity against challenge with homologous virus.

On the same day, the vaccinated steer ED 89, which had been immune when challenged with strain Turkey 323/62 at the start of the experiment, was inoculated at ten sites with a 1/20 suspension of vesicular epithelium from steer ED 88, the last animal in the passage series. By contrast with the homologous virus challenge, although steer ED 89 also possessed SVNT of 1/1024 against virus of strain Turkey 323/62, the 'heterologous' strain produced extensive lesions on the tongue but generalization did not occur. Similarly, steers EH 7 and EC 83, the 1st and 2nd vaccinated animals in the passage series, which had recovered from generalized

infections with virus from the unvaccinated steer EH 6 some 10 weeks previously, were both inoculated at ten sites with a 1/20 suspension of vesicular epithelium from steer ED 88. Steer EH 7, whose SVNT against strain Turkey 323/62 virus was 1/355 at the time of the second infection, developed two primary vesicles and a secondary vesicle appeared on one foot. Steer EC 83, whose SVNT against virus of strain Turkey 323/62 was 1/512, developed a single large vesicle on the tongue and secondary vesicles occurred on the dental pad and on both hind feet.

The fluid and epithelium from the primary vesicle of steer EC 83 were harvested under anaesthesia. CFT on this material demonstrated some fixation of complement in the presence of WRL stock antiserum of Type SAT 1 but no fixation occurred with stock reference sera of FMD virus of the remaining six types. By virtue of its ability to break through the immunity of recovered cattle, this strain, from steer EC 83 was considered to be an extreme subtype variant of Type SAT 1 and was designated strain C³⁴.C¹.

In an attempt to determine whether variation had occurred early or later in the passage series, a 1/10 suspension of the vesicular epithelium from the second infection of steer EC 83 was inoculated into steers EG 94, 95, 96 and 97, which were respectively the 24th–27th cattle in the passage series. No clinical reactions resulted.

Passage and contact infection in cattle possessing waning immunity

To simulate conditions which might arise after vaccination campaigns in the field, four new steers, ED 81–84, were all vaccinated with 15 ml. doses of strain Turkey 323/62 vaccine on 6 May 1963; steers ED 83 and 84 were revaccinated on 27 September 1963. The immunity of the group was allowed to regress naturally until 2 December 1963 and on this date their SVNT against virus of strain Turkey 323/62 were 1/22, 1/22, 1/178 and 1/90 respectively.

Steer ED 81 was inoculated on the tongue with 10–100 ID 50 of virus from steer EH 6 (strain Turkey 323/62). Steer ED 82 was not inoculated but was housed in contact with steer ED 81. Steer ED 83 was inoculated with 10–100 ID 50 of virus from steer ED 88 (strain C³⁴). Steer ED 84 was allowed to remain in contact with steer ED 83.

Both of the inoculated steers developed generalized lesions, although steer ED 83 possessed a SVNT of 1/178 against virus of strain Turkey 323/62. Whilst residual immunity (SVNT 1/22) protected steer ED 82 against contact infection with the homologous strain Turkey 323/62, the heterologous strain C³⁴ was able to spread by contact from steer ED 83 to steer ED 84 and generalized later to two feet, despite a SVNT of 1/90 against virus of the original strain Turkey 323/62. Subsequently, steer ED 82 proved to be fully susceptible to parenteral challenge with strain C³⁴.

Virus titres in the lesions of immunized cattle

It appeared possible that the infectivity titre of virus present in the vesicular epithelium of cattle possessing high antibody titres might be much less than the virus titres of susceptible cattle or of cattle only incompletely immunized. Consequently, fragments of the epithelium were harvested during passage at the 24th

hour after inoculation and the infectivity of samples collected at several passage levels was titrated in mice. The results shown in Table 2 indicate that, even during the early passages, humoral antibody titres exerted little influence on the titre of virus in the vesicles. The virus titres of the primary vesicles of steers ED 51, 52 and 53, in which infection failed to generalize, were $10^{5.9}$, $10^{6.1}$ and $10^{6.2}$ mouse ID 50/ml. respectively. These were not significantly different from those found in the primary lesions of animals in which generalization occurred.

In the later passages, by which time adaptation was well advanced, the virus titre of the epithelium was also independent of the amount of virus inoculated.

Table 2. *FMD virus in tongue epithelium fragments harvested 24 hr. after the infection of partly immunized cattle*

Animal no.	SVNT for strain Turkey 323/62	Dilution of inoculum	Virus titre of epithelium harvested (mouse ID 50/g.)
EH 6	$\leq 1/3$	1/10	$10^{6.125}$
EC 83	1/45	1/10	$10^{6.7}$
EC 89	1/256	1/10	$10^{6.8}$
ED 50	1/45	1/10	$10^{6.4}$
ED 55	1/32	1/10	$10^{7.2}$
EG 69	1/128	1/10	$10^{7.1}$
EG 89	1/32	1/10	$10^{5.9}$
EG 95	1/90	1/10	$10^{6.6}$
EG 97	N.A.	1/1,000	$10^{6.1}$
EG 68	1/64	1/1,000	$10^{5.3}$
ED 87	1/256	1/100,000	$10^{8.0}$
ED 88	1/708	1/100,000	$10^{7.0}$

N.A. = not available.

Serum-virus neutralization tests

Each animal was bled for serum immediately before the inoculation of virus. Virus in filtrates of tongue epithelium from steer EC 83, the 2nd vaccinated animal in the cattle passage series and the 1st in which a significant antibody titre was detectable before infection, was passaged 6 times in pig kidney monolayer cultures and was then used in serum-virus neutralization tests. Virus in filtrates of the 2nd crop of vesicles from steer EC 83 (strain C³⁴.C¹) was passaged similarly in pig kidney monolayers 6 times and was then employed in further serum-virus neutralization tests with all the cattle sera tested previously against the virus isolated from the 1st infection of this animal. As a further check, all sera were also tested against a stock (high PKTC passage) substrain of strain Turkey 323/62.

All except one of the thirty-four serum samples collected from cattle infected later than the 1st infection of steer EC 83 possessed SVNT to virus of strain Turkey 323/62 which were equal to or greater than 1/32, but only the serum of steer ED 88 had an antibody titre of 1/32 against virus of strain C³⁴.C¹ and the sera from thirty-one animals had titres equal to or less than 1/6.

The SVNT of all sera when tested against the stock (high PKTC passage) virus of strain Turkey 323/62 was usually one serial dilution lower than the titre against the 6th PKTC passage. The geometric mean titres of all the serum samples tested against the three virus strains were:

Turkey 323/62 stock	1/40
Turkey 323/62 6th PKTC	1/80
C ³⁴ .C ¹ 6th PKTC	1/4.

The very great difference in the neutralizing titres of the sera of individual cattle against the two strains isolated from steer EC 83 (Table 1, cols. 6 and 7), after both strains had experienced the same number of passages in PKTC, suggests that a considerable degree of subtype variation had occurred during passage in immunized cattle; and the SVNT are consistent with the clinical results only if such variation had occurred.

Reinfection of convalescent guinea-pigs

Virus of the 1st and 2nd strains from steer EC 83 was passaged 3 times in groups of guinea-pigs which were housed in separate rooms. The guinea-pig passaged strains were each inoculated into further groups of guinea-pigs and generalization of infection resulted. On the 28th day after primary infection, the immunity of eight guinea-pigs convalescent from infection with strain 1 was challenged by intradermal inoculation of virus of the guinea-pig passaged homologous strain; no lesions resulted. On the same day, ten guinea-pigs convalescent from infection with strain 2, together with five susceptible controls, were subjected to challenge with virus of the guinea-pig passaged strain 1; all developed generalized lesions.

Complement-fixation tests

Virus of the 6th PKTC passages of the two strains isolated from steer EC 83 were each passaged twice on the plantar pads of guinea-pigs. The 'adapted' viruses were used to immunize further groups of guinea-pigs, and pooled sera from these groups were employed in CFT against the homologous and heterologous viruses derived from the respective 6th PKTC. The complement-fixation ratios were determined and the CFP for the two strains was found to be 0.28. Because the CFP of the two strains was less than 0.5 they may be regarded as antigenically different.

Similarly, CFP with the viruses of strains 1 and 2 from steer EC 83 were determined for all available stock strains of Type SAT 1. The results, which are expressed as histograms in Fig. 1, indicate that strain 1 was identical with the WRL stock strains Turkey 323/62 and Greece 7/62, and it also possessed minor antigenic relationships with several other strains. Strain 2, however, possessed only a minor antigenic relationship with strain 1 and was devoid of significant relationship to the other strains.

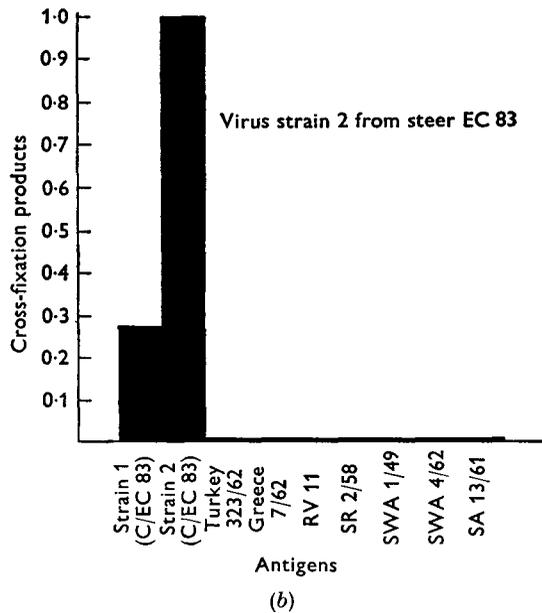
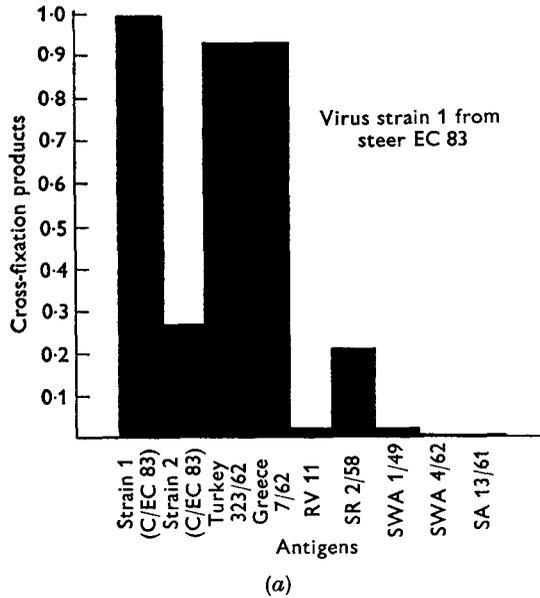


Fig. 1. Histogram of cross-fixation products with SAT 1 strains of FMD.

DISCUSSION

During a major epizootic in a fully susceptible population, FMD appears often to infect very large numbers of animals without showing marked modification of the characteristics of the causal strain of virus; thus, in 1962–63, although FMD of Type SAT 1 spread from Bahrein in the southern part of the Persian Gulf through Israel and Turkey to the borders of Greece, the serological characteristics

of the late strains from Greece resemble closely those of the early strains from Israel. It is not possible to state unequivocally that FMD of Type SAT 1 had never occurred in the Middle East before 1962 but it is very probable that few, if any, of the cattle in the area had previously experienced a virus of this type.

By contrast, a number of markedly *different* strains have been isolated in recent years in Southern Africa, where FMD of Type SAT 1 is endemic. For example, strains SA 13/61 and SWA 40/61, both isolated in the same year, though showing some antigenic resemblance were nevertheless demonstrably different from one another and differed to a much greater extent from the 'older' strain, RV11 (Hyslop *et al.* 1963), and also from the Middle East strain Isr. 4/62 (Hyslop, unpublished result). These observations are consistent with a widely accepted belief that variant strains of many viruses pathogenic for animals and man tend to become dominant when either vaccination or previous exposure to endemic infection causes resistance to increase in the general 'host' population.

Whether in the case of FMD this effect results *in vivo* solely from the presence of high serum antibody titres in the animal population or is caused by the interaction of several factors remains unknown; but the influence of serum antibody was illustrated *in vitro* by the emergence of immunologically distinct substrains from strains of FMD virus propagated in PKTC monolayers maintained in media containing progressively increasing concentrations of homologous cattle or guinea-pig antiserum (unpublished results).

In the present experiment, the antibody titres of successive cattle did not increase uniformly. Although at the start of the experiment the graded doses of vaccine probably produced an 'immunity gradient' between the 1st animal of the series and the last, the natural regression in SVNT which occurred during the period of passage largely offset the increase in dosage. Nevertheless, after the 1st passage, SVNT always indicated a marginal or clearly protective degree of immunity against the original strain of virus, and the last eight animals possessed SVNT great enough to have protected them against generalization of virus of the 'parent' strain.

The experimental data do not reveal clearly the stage at which the emergence of the variant strain was most rapid. It is particularly noteworthy, however, that by the 5th passage the virus was able to generalize despite a SVNT of 1/256, and generalization occurred at the 7th passage although the animal possessed a SVNT of 1/355—a titre which is very frequently sufficient to protect against any clinical response to challenge whatsoever. The probability that some degree of adaptation continued throughout the greater part of the passage series is indicated by the CFP of the virus samples isolated at the 2nd, 10th and 35th passages; thus, although the CFP had fallen to 0.47 by the 10th passage, a further decrease to 0.27 occurred during the subsequent twenty-five passages. Furthermore, the inability of the strain of virus isolated from the 35th animal of the passage series to reinfect the 25th, 26th or the 27th animals suggests that variation between the 1st and the 25th passages may have been very much greater than that between the 25th and 35th passages.

It seems possible that the change in antigenic structure might have been more

rapid if epithelium had been harvested routinely from lesions which had generalized to the feet instead of principally from lesions on the tongue. However, if the former method had been adopted it would have been necessary to delay each passage until foot lesions appeared and to accept the risk, at each passage, that generalization might not occur; also the recovery of adequate amounts of material from small and often ruptured foot vesicles presents obvious difficulties. The possible danger of dissemination of variant strains from the foot lesions of naturally infected cattle was demonstrated by titrating epithelium harvested from the 15th serial passage (steer ED 55), by which passage demonstrable variation had already occurred; the titre of the tongue epithelium of this animal was $10^{7.2}$ mouse ID 50/g. and that of the foot epithelium was $\geq 10^{6.75}$ mouse ID 50/g.

The results shown in Table 2 indicate that, irrespective of titres of humoral antibody against the 'parent' strain of virus, cattle with primary vesicles caused by emergent variants were likely to be highly infective; as much virus was detected in such lesions at the 24th hour as was present in 24 hr. samples collected in this and in other experiments from the lesions of fully susceptible cattle. No attempt was made to transmit the emergent strain by contact until it had been passaged in immunized cattle 28 times but by this passage the strain was able, by contact infection, to overcome the resistance of steer ED 99, which had been inoculated twice with the standard 15 ml. dose of vaccine; two other animals having the same vaccination history possessed SVNT which were high enough to protect against the 'parent' strain. Contact infection also occurred between vaccinated steers ED 83 and ED 84, when the strain had been passaged 34 times in vaccinated cattle.

The capacity of the variant strain to infect cattle possessing SVNT as high as 1/355, 1/512 and 1/708 against virus of the 'parent' strain suggests a very considerable degree of difference in antigenic structure between the two strains, and the ability of the 'parent' strain to reinfect guinea-pigs convalescent from infection with virus of the variant strain provides additional evidence of a dissimilarity of significant magnitude.

The mean SVNT of the sera of all cattle tested, 1/80 against the 6th PKTC of virus of strain 1 of steer EC 83 but only 1/4 against the 6th PKTC of virus of strain 2, provided supplementary evidence of change during serial passage and confirmed the difference in antigenic constitution revealed by the CF tests.

Whether the antigenic change was a gradual process throughout the experiment or was a stepwise progression during certain phases is not revealed by the CFP. Nevertheless, the CFP of two strains from steer EC 83 shows an obvious difference between the strains. It appeared possible that the influence of humoral antibody might have caused the strain to change until it resembled one of the older field strains of Type SAT 1. Fig. 1 demonstrates that a change of this nature did not result; indeed, in this particular instance, a well-marked loss of antigenic components common to strain Turkey 323/62 and the other stock strains of Type SAT 1 appears to have occurred during passage. CF tests with sera of the WRL stock strains of types other than SAT 1 failed to reveal a significant degree of fixation of complement. The possibility that additional passages in highly resistant

cattle might result in the emergence of a strain possessing the characteristics either of a different serological type or of an entirely new type cannot be excluded without further investigation. The present observations suggest one way in which subtype variant strains may arise in areas where the disease is endemic or where the herd immunity induced by vaccination is allowed to become dangerously low.

SUMMARY

Foot-and-mouth disease virus of Type SAT 1 (strain Turkey 323/62) was passaged serially 34 times in cattle previously vaccinated with increasing doses of formol-treated vaccine of the homologous strain. Primary vesicles developed in all the partly immunized animals and secondary lesions occurred in the majority. Virus from the 34th passage was capable of reinfecting a steer only 76 days after primary infection early in the passage series. Virus isolated from the second infection of this animal differed from that isolated from the primary infection in complement-fixing properties and in sensitivity to antiserum, and these differences were of a degree indicative of subtype variation.

The variant strain was transmissible by contact, and virus titres in tongue and foot lesions of partly immunized animals were of the same order as those encountered in susceptible cattle.

These observations suggest one way in which variant strains may arise in the field.

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REFERENCES

- BRADISH, C. J. & BROOKSBY, J. B. (1960). Complement fixation studies of the specificity of the interactions between components of the virus system of foot-and-mouth disease and its antibodies. *J. gen. Microbiol.* **22**, 405.
- BRADISH, C. J., BROOKSBY, J. B. & TSUBAHARA, H. (1960). The complement fixation test in studies of components of the virus system of foot-and-mouth disease and its antibodies. *J. gen. Microbiol.* **22**, 392.
- BROOKSBY, J. B. (1952). The technique of complement fixation in foot-and-mouth disease research. *A.R.C. Rep. Ser.* no. 12.
- FRENKEL, H. S. (1949). Histologic changes in explanted bovine epithelial tongue tissue infected with the virus of foot-and-mouth disease. *Am. J. vet. Res.* **10**, 142.
- FRENKEL, H. S. (1951). Research on foot-and-mouth disease. III. The cultivation of virus on a practical scale. *Am. J. vet. Res.* **12**, 187.
- FRENKEL, H. S. (1953). Recherches sur la fièvre aphteuse. IV. *Bull. Off. int. Épizoot.* **39**, 91.
- HYSLOP, N. ST G., DAVIE, J. & CARTER, S. P. (1963). Antigenic differences between strains of foot-and-mouth disease virus of type SAT 1. *J. Hyg., Camb.*, **61**, 217.
- KÄRBER, G. (1931). Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche. *Arch. exp. Path. Pharmak.* **162**, 480.
- MARTIN, W. B. & CHAPMAN, W. G. (1961). The tissue culture colour test for assaying the virus and neutralizing antibody of foot-and-mouth disease and its application to the measurement of immunity in cattle. *Res. vet. Sci.* **2**, 53.
- SKINNER, H. H. (1951). Propagation of strains of foot-and-mouth disease virus in unweaned white mice. *Proc. R. Soc. Med.* **44**, 1041.