J. Hyg., Camb. (1966), **64**, 397
Printed in Great Britain

Isolation of a variant strain of foot-and-mouth disease virus (type O) during passage in partly immunized cattle

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(*Received* 16 *April* 1966)

Evidence of the existence of different subtypes within the seven principal types of foot-and-mouth disease (FMD) virus was described by Hyslop, Davie & Carter (1963). Recently we reported the isolation of an immunologically distinct variant strain of virus of type SAT 1, which evolved when strain Tur. 323/62 was passaged serially through thirty-four partly resistant cattle (Hyslop & Fagg, 1965). The variant was distinct from all the other subtype strains of type SAT 1 against which it was tested, and these observations provided experimental support for theories on the mechanism by which new subtypes might arise during outbreaks of FMD in the field.

The present report records a similar experiment, using a virus strain of type O, in which a new variant evolved after fewer passages than were required for the isolation of the variant strain of type SAT 1.

MATERIALS AND METHODS

Virus

FMD virus of type O (strain Israel 1/63), isolated during a recent epizootic, was inoculated into susceptible cattle at Pirbright and subsequently was passaged serially in further cattle which had been partly immunized with a vaccine prepared from virus of the homologous strain. The serial passage followed the procedure described by Hyslop & Fagg (1965) and the sequence is shown in Table 1.

Vaccine

The groups of cattle were vaccinated with one or several graduated doses of inactivated vaccine prepared by the method described by Morrow, Hyslop & Buckley (1966). A concurrent experiment demonstrated that the vaccine (batch 6405) was of high potency.

Experimental procedure

The source and maintenance of cattle, the methods used for titration of virus and for the serological tests were the same as the procedures described in the previous report (Hyslop & Fagg, 1965).

Table 1. *Passage of FMD virus of strain Israel* 1/63 *in partly immunized cattle*

Virus passage no.	Animal no.	Vaccination history (doses)	SVNT of sera against virus of: Original strain	SVNT of sera against virus of: EQ 33 strain	Virus inoculated: Source	Virus inoculated: Dilution	Clinical result of inoculation
A	EQ 90	Unvaccinated	N.A.	N.A.	Field	10^{-1}	T, 4F
	EQ 91	Unvaccinated	N.A.	N.A.	Field	10^{-1}	T, D, 4F
B	EQ 92	Unvaccinated	N.A.	N.A.	Tongue EQ 90/91	—	T, 4F
	EQ 93	Unvaccinated	N.A.	N.A.	Tongue EQ 90/91	—	T, M, 4F
1	EP 72	1 × 2·0 ml.	1/45	1/32	Tongue EQ 90/91	10^{-2}	T, 1F
	EP 73	1 × 2·0 ml.	N.A.	N.A.	In contact	—	
1*a*	EQ 76	1 × 15 ml.	1/90	1/45	Tongue EQ 90/91	10^{-2}	T
2	EP 74	1 × 2·0 ml.	N.A.	N.A.	Tongue EP 72	10^{-1}	T, 2F
3	EP 75	1 × 2·0 ml.	N.A.	N.A.	Tongue EP 74	10^{-2}	T, D, 4F
4	EP 76	1 × 5·0 ml.	1/90	1/45	Foot EP 75	10^{-3}	T, 1F
5	EP 77	1 × 5·0 ml.	1/6	1/6	Tongue EP 76	10^{-4}	T, 4F
6	EP 78	1 × 5·0 ml.	1/90	1/90	Tongue EP 77	10^{-5}	T, 2F
7	EP 79	1 × 5·0 ml.	1/32	1/22	Tongue EP 78	10^{-6}	T, D, 4F
—	EP 80	1 × 10 ml.	—	—	Tongue EP 79	10^{-7}	No reaction
8	EP 80	—	N.A.	N.A.	Tongue EP 79	10^{-6}	T, 4F
—	EP 81	1 × 10 ml.	—	—	Tongue EP 79	10^{-7}	No reaction
9*a*	EP 81	—	1/128	1/90	Tongue EP 80	10^{-6}	T, 1F
—	EP 82	1 × 10 ml.	—	—	Tongue EP 81	10^{-7}	No reaction
10*a*	EP 82	—	1/45	1/32	Tongue EP 81	10^{-6}	T, 3F
11*a*	EP 83	1 × 10 ml.	N.A.	1/6	Tongue EP 82	10^{-6}	T, 1F
—	EP 84	1 × 15 ml.	1/2048	1/355	Tongue EP 83	10^{-7}	No reaction
—	EP 84	—	—	—	Tongue EP 83	10^{-6}	No reaction
—	EP 84	—	—	—	Tongue EP 83	10^{-5}	No reaction
9	EQ 28	s.I. + 1 × 15 ml.	1/178	1/90	Tongue EP 80	10^{-6}	T, 1F
10	EQ 29	s.I. + 1 × 15 ml.	1/45	1/16	Tongue EQ 28	10^{-6}	T, 4F
—	EQ 30	s.I. + 2 × 15 ml.	—	—	Tongue EQ 29	10^{-6}	No reaction
11	EQ 30	—	1/1400	1/128	Tongue EQ 29	10^{-4}	T, 2F
12	EQ 31	2 × 15 ml.	1/90	1/45	Tongue EQ 30	10^{-4}	T, 1F
13	EQ 32	2 × 20 ml.	1/178	1/90	Tongue EQ 31	10^{-5}	T, 3F
14	EQ 33	2 × 20 ml.	1/355	1/128	Tongue EQ 32	10^{-5}	T, 1F
15	EQ 90	Recovered 34 days after passage A	1/512	1/128	Tongue EQ 33	$10^{-1·3}$	T
15	EQ 91		1/2800	1/512	Tongue EQ 33	$10^{-1·3}$	T
—	EQ 92	Recovered 33 days after passage B	1/1400	1/355	Tongue EQ 33	$10^{-1·3}$	No reaction
—	EQ 72	1 × 15 ml. challenged, 35 days convalescent	1/355	1/90	Tongue EQ 32	$10^{-1·3}$	(TE)
16	EQ 73		1/1024	1/256	Tongue EQ 91 (passage 15)	$10^{-1·3}$	T

s.I., Sensitizing inoculation; SVNT, virus neutralizing titre of serum; T, primary vesicles on tongue; I lesion on dental pad; M, secondary vesicle on muzzle; 1F–4F, secondary vesicles on 1 or more feet; N.A result not available; TE, erosive lesion on tongue.

https://doi.org/10.1017/S0022172400040699 Published online by Cambridge University Press

RESULTS

Clinical results in cattle

Passage of virus in vaccinated cattle

Groups of cattle were vaccinated subcutaneously with strain Isr. 1/63 vaccine in doses of 2·0 ml., 5·0 ml., 10·0 ml. or 15·0 ml. Other animals, some of which had been sensitized during innocuity tests of FMD vaccines, were vaccinated with either one or two doses of 15·0 ml. or with two doses of 20·0 ml.

In a concurrent experiment, a single 15·0 ml. dose of the vaccine protected all of ten steers (EQ 70–79) against generalization of lesions after challenge by inoculation of the tongue with the homologous virus.

Virus of the field strain Isr. 1/63 was passaged in the tongues of steers EQ 90 and EQ 91. A filtrate of the harvested material had a titre of $10^{6\cdot8}$ ID 50/ml. when titrated in two cattle by the method of Henderson (1952). One of these animals died as a result of generalized FMD.

On the 21st day after vaccination, a virus filtrate (diluted to contain $10^{3\cdot0}$ ID50/ml.) was inoculated into the lingual mucosa of steer EP 72. Vesicles appeared on the tongue after about 40 hr. and the overlying epithelium, harvested under general anaesthesia, was found to have a virus titre of $10^{9\cdot0}$ mouse ID50/g. Steer EP 73, vaccinated at the same time as steer EP 72 and housed in contact, did not develop FMD. Material from steer EP 72 was used to continue the serial passage in vaccinated steers shown in Table 1.

To ensure that the strain was capable of generalizing in the presence of antiserum and was not merely being passaged mechanically, virus harvested from the foot lesions of the third animal in the series was used for the 4th passage.

Because of individual variations in immunizability (Hyslop, 1966), a smooth 'gradient' of serum-virus neutralization titres (SVNT) was not established but the majority of the animals in the latter part of the series possessed SVNT which might have been expected to prevent generalization of infection to the feet if no change had occurred in the antigenic constitution of the virus strain.

Between the 2nd and 7th passages the dilution of the inoculum was increased tenfold at each passage but, when an attempt was made to passage the strain at a dilution of 10^{-7} for the 8th serial passage, steer EP 80 failed to react. On the following day, however, inoculation of the same virus suspension diluted only 10^{-6} resulted in infection and lesions generalized to all feet. Probably as a result of the balance between the infectivity of the inoculum and the SVNT of the recipient, by the 11th passage it was necessary again to decrease the dilution of the inoculum. The last two passages in vaccinated cattle were made at a dilution of 10^{-5}.

Table 2 indicates that the amount of virus in the inoculum exerted little influence on the titre of the resultant vesicles but the high SVNT of 1/355 at the 14th passage probably reduced the virus titre about 100-fold; if a change in antigenic structure had not occurred, this SVNT might well have been sufficient to inhibit the development of lesions completely. Infection generalized after each successful passage in the vaccinated cattle.

Reinfection of recovered cattle

After the 14th passage in semi-resistant cattle a 1/20 suspension of triturated tongue epithelium from the last animal (steer EQ 33) was inoculated at five sites in the lingual mucosa of each of the two steers EQ 90 and EQ 91; these animals had been infected with field virus at the start of the experiment. Although the animals had recently recovered from experimental infection, EQ 91 developed

Table 2. *Relationship between dilution of inoculum, antibody titre and titre of virus in tongue lesions of semi-resistant cattle infected with FMD virus*

Passage no.	Dilution of inoculum	SVNT (against original strain)	Virus titre of tongue epithelium (mouse ID 50/g.)
1	10^{-2}	1/45	$10^{9.0}$
3	10^{-2}	N.A.	$10^{9.5}$*
4	10^{-3}	1/90	$10^{10.0}$
6	10^{-5}	1/90	$10^{10.0}$
7	10^{-6}	1/32	$10^{10.5}$
10	10^{-6}	1/45	$10^{9.1}$
12	10^{-4}	1/90	$10^{9.5}$
14	10^{-5}	1/355	$10^{7.5}$

* Titre of epithelium from foot: $10^{5.0}$.
N.A. Result not available.

one and EQ 90 two small lesions at the site of inoculation. The same inoculum failed to cause lesions in steer EQ 92, although its SVNT against virus of the original strain was intermediate between those of steers EQ 90 and EQ 91. Similar material from the penultimate animal of the vaccinated group, when inoculated into the tongue of steer EQ 72 (vaccinated and then challenged 35 days previously in the course of the concurrent vaccine potency experiment), evoked an erosive lesion on the tongue which progressed to a shallow ulcer. A 1/20 suspension of tongue epithelium from the second reaction of the recovered steer EQ 91, when inoculated into the tongue of steer EQ 73 (vaccinated and challenged during the same experiment as EQ 72), resulted in the development of a single small vesicle of about 3 mm. diameter.

Serum-virus neutralization tests

All cattle were bled for serum immediately before experimental infection. The results of serum-virus neutralization tests against virus of the original strain and against the variant strain isolated from the last steer of the vaccinated group are shown in Table 1.

At the start of the experiment a SVNT of 1/90 against the original strain was associated with protection against that strain (steer EQ 76). Later in the series much greater titres were found in animals which failed to resist generalization.

The mean antibody titre of all serum samples was 1/190 with virus of the original strain and 1/68 with virus of the strain isolated from steer EQ 33 at the end of the serial passage. This indicates that the two strains of virus are different in antigenic structure.

Complement fixation tests

Part of the vesicular epithelium harvested from the tongues of infected cattle was triturated and used as antigen in complement fixation tests employing the stock type antisera prepared by the World Reference Laboratory (W.R.L.). Other samples of epithelium were used in tests for which strain-specific antisera were prepared.

Typing tests

Epithelium from the tongue vesicles of the donor steers fixed complement strongly in the presence of W.R.L. antiserum of type O. When the strain was sub-inoculated into steers EQ 92 and EQ 93, complement fixation tests on epithelium again revealed only virus of type O; one of these animals (steer EQ 93) died, however, and material from the heart lesions found at necropsy fixed complement strongly with W.R.L. serum of type O and also showed slight fixation with stock sera of types Asia 1 and C. Thereafter, traces of fixation with sera of type C were detected in epithelium samples collected from the partly immunized cattle at the 1st, 8th, 9th and 13th pasages. The O/C ratio was determined for virus of the 1st, 2nd, 3rd, 9th, 12th and 14th passages. The cross-reactions were not consistent and there was no evidence of a trend towards greater similarity to type C. Virus from the 1st passage fixed a trace of complement in the presence of antiserum of type Asia 1 but no complement was fixed with serum of this type by virus isolated after the last passage.

Table 3. *Cross-fixation products of FMD virus samples isolated during passage in partly immunized cattle*

	Virus from animal no.:			
	EQ 90	EP 72	EP 77	EQ 33
EQ 90	1·00	1·00	0·54	0·32
EP 72	1·00	1·00	0·52	0·38
EP 77	0·54	0·52	1·00	0·44
EQ 33	0·32	0·38	0·44	1·00

Tests using strain-specific sera

Guinea-pig antisera were prepared with virus derived from the tongue lesions of steers EQ 90 and EQ 91, the unvaccinated cattle, also from steer EP 72, the first partly immune animal, and from steers EP 77 and EQ 33, the 5th and the last of the partly immune cattle, respectively.

The cross-fixation products of the various serum-virus pairs are shown in Table 3. A considerable degree of variation was found after five passages of strain Israel 1/63 in vaccinated cattle. Although the change between the 5th and the 14th passages was smaller than that detected after the first five passages, the difference between the original strain and that isolated after the 14th serial passage was very great.

DISCUSSION

At the first passage of strain Israel 1/63 in partly immunized cattle, the virus was just able to generalize to a single foot of a steer which possessed a SVNT of 1/45 but it failed to generalize in an animal whose SVNT was 1/90. At the 4th and 6th passages, generalization occurred in cattle having SVNT of 1/90 and, by the 9th passage, the virus was able to generalize despite SVNT of 1/128 and 1/178, respectively. This developing ability to generalize suggests that the virus had adapted to some extent to its new and adverse environment in semi-resistant cattle; the change apparently occurred quite rapidly. Additional evidence of an early modification of antigenic structure is afforded by a decrease in the cross-fixation product (C.F.P.) between the first and the fifth passages (Table 3). However, the difference in C.F.P. between the first and second passages was not significant and this renders it less probable that the change resulted solely from differential inhibition within an initially very heterogeneous virus population.

By the 14th serial passage the C.F.P. with virus of the first passage clearly indicated that a distinct strain had evolved. Furthermore, when the cattle were infected, each possessed antibody against virus of the vaccine strain and the mean SVNT of all serum samples was 1/190: the lower mean SVNT of 1/68 of the same serum samples, when tested against virus isolated from the 14th animal in the passage series (steer EQ 33), provides still further evidence of antigenic variation in the virus.

Recovered cattle generally have been found to resist reinfection by intramuscular inoculation of large amounts of virus of the homologous strain for periods exceeding two years; inoculation of the same virus into the lingual mucosa will cause small primary lesions some months after clinical recovery, though such lesions are usually limited in number and generalization of infection does not occur. Inoculation of virus of heterologous subtypes by the latter route may evoke both primary and secondary vesicles after shorter periods. By the 14th serial passage in partly immunized cattle, the variant of strain Israel 1/63 had altered sufficiently to cause small primary vesicles when inoculated into the tongues of steers EQ 90 and EQ 91. It is noteworthy that reinfection of these animals was achieved only 34 days after their original infection with strain Israel 1/63. At the time of reinfection, steer EQ 91 possessed a SVNT of 1/2800. Steer EQ 92 failed to react to the same inoculum. Despite a SVNT of 1/1024, steer EQ 73 (vaccinated and then challenged with strain Israel 1/63, to which it reacted 35 days previously) developed primary vesicles when it was inoculated again with virus from the second infection of steer EQ 91.

After experimental infection, the virus titre of the epithelium covering the vesicles of semi-resistant cattle was found to be very high and this titre was affected little either by the dilution of the inoculum or by the titre of antibody in the subject's serum. Samples of saliva from these cattle were highly infective and, undoubtedly, such cattle would have been capable of disseminating FMD under field conditions.

In spite of the great infectivity of the lesions caused by the first passage of strain

Israel 1/63 in vaccinated steers EQ 72 and EQ 76, a third vaccinated steer (EP 73), housed in contact with them, failed to develop clinical signs of FMD and was apparently protected against contact infection by a single 2·0 ml. dose of vaccine. At the end of the experiment, however, this animal was found to have developed a SVNT of 1/708 against virus of the original strain; it must be assumed, therefore, to have experienced an inapparent infection. A similar inapparent infection in vaccinated steer EP 84 evoked a SVNT of 1/2048, a value greatly in excess of vaccinal titres, and this was sufficient to protect the animal against infection when the time arrived for it to be inoculated with virus of the 11th serial passage. Inapparent infections of this nature have been observed in other experiments in which vaccinated cattle have been exposed to infection by contact. It is possible, therefore, by leaping ahead of any emergent strains, that 'parent' strains in the field may occasionally reinforce vaccinal immunity to a level at which dissemination of emergent strains may be inhibited or blocked completely. If such a sequence of events occurs frequently in the field, the emergence of variants may depend upon a delicate balance of numerous factors and the outcome (i.e. dominance or otherwise of the variant) may be largely fortuitous.

The results of the present investigations show certain contrasts with those of a previous experiment employing strain Tur. 323/62 of type SAT 1, but the differences may be attributable to modifications of technique as much as to differences in the strains of virus. During serial passage of strain Israel 1/63, the inoculum was diluted progressively from the 2nd to the 7th passage, whereas in the earlier experiment the inoculum was diluted progressively only after the 26th passage. Furthermore, in the second experiment the total number of serial passages to which strain Israel 1/63 was subjected was lower than that for strain Tur. 323/62. Nevertheless, the results of these investigations confirm our previous observations and demonstrate that antigenic lability is not confined to FMD virus of a single strain or type. It is also evident that, under the adverse conditions of serial propagation in semi-resistant hosts, such lability may be manifested after only a few (less than ten) passages. The difference between the mean SVNT of all the post vaccinal sera, when tested against virus of the original and the variant strains, was less in the case of the type O strains (mean SVNT 1/190 and 1/68, respectively) than for the strains of type SAT 1 (mean SVNT 1/80 and 1/4). Similarly, although complement-fixation tests may be quantitatively less reliable than neutralization tests, the C.F.P. of the original and the variant strains was slightly greater (C.F.P. 0·325) after 14 passages of strain Israel 1/63 than after 34 passages of strain Tur. 323/62 (C.F.P. 0·27). It is probable, therefore, that strain Israel 1/63 was modified to a somewhat lesser extent than was strain Tur. 323/62.

The original sample of bovine tongue epithelium received from the field outbreak, from which strain Israel 1/63 was subsequently isolated, not only fixed complement strongly in the presence of W.R.L. antiserum of type O but also showed very slight cross-fixation with antiserum of type C and a trace of cross-fixation with antiserum of type Asia 1. Complement fixation tests on virus isolated from the saliva of reacting cattle also showed traces of cross-fixation with antiserum of type C. At the outset it appeared possible that passage of the virus in cattle

whose serum contained antibody strongly inhibitory to virus of type O might evoke a change towards type C, and complement fixation tests revealed slight cross-fixation with type C intermittently throughout the experiment. It is noteworthy that FMD virus of type C has not been recorded in Israel. However, at no stage in the experiment did the passaged strain show a greater affinity for type C than for type O and the principal changes occurred only in antigenic constituents specific for type O. Whether or not subsequent serial passage in cattle partly immunized against the *variant* strain might lead eventually to the evolution of virus of one of the other types remains a matter for conjecture.

New strains have been recovered during passage of FMD virus in monolayer cultures of porcine kidney cells in the presence of strain-homologous antiserum (Hyslop, 1965).

SUMMARY

Foot-and-mouth disease virus of type O (strain Israel 1/63) was passaged serially fourteen times in cattle previously vaccinated with increasing doses of formol-treated vaccine of the homologous strain. Primary and secondary lesions developed in the majority of the animals. Virus from the 14th passage, which proved to be capable of reinfecting animals only 34 and 35 days after infection with the original strain, differed from the original strain in complement-fixing properties and in sensitivity to antiserum. These changes were of a degree indicative of subtype variation.

Virus titres in the saliva, in the tongue vesicles and in the foot vesicles of partly resistant cattle were very great and would have constituted a danger to other stock under field conditions.

The experimental data confirm previous observations on the infection of partly resistant cattle and on the isolation of variant strains during passage of FMD virus in tissue cultures containing specific antiserum.

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