

MULTIPLICATION OF AN ATTENUATED RV11 STRAIN (TYPE SAT1₂) OF FOOT-AND-MOUTH DISEASE VIRUS IN SHEEP

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INTRODUCTION

The development of attenuated virus vaccines for the control of foot-and-mouth disease has included studies of their pathogenicity and immunogenicity for cattle and pigs. There are, however, few references in the literature on the effect of either the original virulent or the attenuated virus strains in sheep.

Investigations at Pirbright have shown that strains of virus which are attenuated and immunogenic for cattle were also attenuated for sheep and produced a good immune response (Report, 1964). The work of Geering & Pay (1963) included studies with the RV11 vaccine strain, which at a high dosage level caused no post vaccinal reactions in a group of twenty four-month old lambs. Examination of the blood and saliva of ten of these lambs during the first week after vaccination, revealed a viraemia in six, while in four of them the saliva was also infective. The neutralizing antibody titres were higher than in cattle. In a preliminary report by Burrows, Geering, Mowat & Skinner (1963) it was shown that sheep which had been immunized with three dosage levels of a modified strain 119 *mo C* type A₁₂ vaccine, developed, neutralizing antibodies within six to thirteen days after vaccination, even when the infectivity of the vaccine was reduced 1,000-fold.

Reports have been made of the field use of the RV11 strain vaccine in 73,000 sheep in South West Africa (Galloway, 1962; Viljoen, 1964) and in 59,000 sheep in Israel, (Report, 1964; Nobel, Neuman & Ripplin, 1963). In both instances under different environmental conditions, these authors described a low incidence of muscular and central nervous disturbance following vaccination. In a small percentage of both adult and young sheep this condition assumed the form of a myositis, followed by a non-purulent lymphocytic encephalitis.

The object of the present investigation was to continue the study of the behaviour of the modified strain RV11 (SAT1₂) in sheep and at the same time to attempt the reproduction of these undesirable side reactions under controlled laboratory conditions.

MATERIALS AND METHODS

Virus strain

The RV11 strain of SAT1 virus modified by serial passage in adult mice (Mowat, 1964) was used. A pool was prepared from stored samples of five batches of vaccine which had been previously used in the field in sheep in South West Africa. This pool when titrated in six-day old mice by the intraperitoneal route, immediately after the inoculation of the experimental animals, gave an endpoint of $10^{7.25}LD_{50}$ per ml. This value indicated that the loss of infectivity during storage at $-20^{\circ}C$ for $3\frac{1}{2}$ years had been minimal (Hollom, Knight & Skinner, 1962).

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Sheep

Experimental animals were drawn from a group of young adult Cheviot sheep varying in age from 15 to 18 months. These animals were taken off pasture in good condition and held in strict isolation until required.

Method of infection

(i) For studying the *in vivo* multiplication of the virus India ink (G. T. Gurr, Ltd.), which had been previously held at 37° C for 12 hours to remove volatile solvents, was added to the virus suspension to give a final concentration of 2 per cent. A group of eight sheep (Group 1) was inoculated into the infraspinatus muscle with 1 ml of vaccine at a site 1 in. below and 1 in. posterior to the supraspinous process of the scapula on the left side. The needle (No. 19 gauge) was pushed into the substance of the muscle until halted by the surface of the scapula, whereupon it was slightly withdrawn and the inoculation completed.

(ii) In an attempt to reproduce nervous symptoms, four more groups of sheep were each inoculated by other routes with vaccine which had been diluted with 0·04M phosphate buffer pH 7·4 as indicated. Where necessary, inoculations were carried out under light general anaesthesia after food and water had been withdrawn for a period of 15 hours.

Group 2 numbering four sheep, received 0·5 ml of a 1:100 dilution of vaccine instilled to a depth of 2½ to 3 in. into each nostril through a fine polythene canula. After administration the head was held back for one minute to facilitate infection of the mucous membranes of the turbinates.

Group 3, also consisting of four sheep, was injected into the epidural space at the lumbo-sacral junction with 1 ml of the 1:100 dilution of the vaccine. The vaccine was diluted to reduce the irritant effect of the glycerine.

Group 4, comprising six sheep, was injected with 1 ml of the undiluted vaccine intramuscularly into the left infraspinatus muscle, followed by the administration of 0·5 ml of a 2 per cent starch solution intracerebrally. The starch solution was injected into the cerebrum after trephining the frontal bone. Spastic muscular movements indicated successful inoculation of the brain.

Finally, Group 5 numbering nine sheep served as vaccinated controls and received 1 ml of undiluted vaccine intramuscularly into the left infraspinatus muscle.

Clinical examination

After inoculation the rectal temperatures of all the experimental animals were taken every day before feeding. The animals were also examined for any signs of disease or alteration in normal behaviour.

Collection of specimens

(i) In order to determine the progress of virus multiplication, one animal was selected at random from Group 1 at 24 hour intervals following inoculation. After the withdrawal of blood and saliva as described below, the animal was stunned and the cervical vessels severed. When bleeding had ceased, the skin was reflected from the left side of the carcass and from each limb as required. With frequent changes to sterile sets of instruments, the various tissue samples were systematically removed.

Particular care was taken with the dissection of the stained muscle tissue at the inoculation site in order to avoid contamination of the surrounding tissues. Tongue epithelium was obtained by scraping the dorsum of the tongue with a sterile scalpel blade.

(ii) **Blood:** At intervals after inoculation samples of blood were withdrawn from the jugular vein and stored at 4° C without preservative. Within three hours of collection the serum was separated and used for the assay of virus.

(iii) **Saliva:** A 2-in. length of $\frac{1}{8}$ -in. bore polythene tubing was attached to a 2 ml syringe and placed alternatively beneath the tongue and between the cheek and gum. By gentle aspiration 0.5 ml to 1 ml of saliva was withdrawn and then added to an equal volume of chilled 0.04M phosphate buffer of pH 7.4. These samples were kept for a minimum period at 4° C until the assay for infectivity.

(iv) **Blood samples** from Groups 2 to 5 were collected at the commencement of the experiment and on the 24th and 105th days when observations were terminated. Samples from Group 5 were also collected 6, 10, 14 and 18 days post vaccination.

Virus assay

With the minimum of delay each tissue sample was cut into small pieces and placed in a weighed jar containing 200 ml of a prechilled (—20° C) solution of equal parts of 0.04M phosphate buffer and glycerine at pH 7.4. After a second weighing to determine the actual amount of tissue collected, the material was finely dispersed in a high speed mixer-emulsifier (Silverson Machines, Ltd.) and a 5 ml sample taken for clarification by centrifugation at 300 rev./min. In the case of small tissue samples such as epithelial scrapings, the volume of buffered glycerine was reduced accordingly and an estimated 1:5 to 1:10 dilution was prepared.

The undiluted supernatant fluid as well as serial ten-fold dilutions thereof were inoculated intraperitoneally in 0.03 ml amounts into groups of four Pirbright "P" strain unweaned mice. Fifty per cent titration end-points, calculated by the method of Reed & Muench (1938), were corrected to give the actual infectivity per gram of tissue.

Blood and saliva samples were similarly assayed for infective virus in suckling mice.

Virus neutralization tests

The presence of neutralizing antibodies in the serum samples was determined by mixing a series of tenfold dilutions of homologous strain virus with an equal volume of a 1:5 dilution of heat-inactivated (56° for 30 min.) serum. The serum-virus mixtures were incubated at 37° C for one hour and 0.03 ml amounts of each mixture were inoculated intraperitoneally into groups of five unweaned mice. The results were recorded as the neutralizing indices of the diluted serum.

RESULTS

Fate of the modified virus after inoculation

The assay of virus in the tissues of each animal killed at 24 hour intervals, is given in Table 1.

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TABLE 1.—Assay of infective virus in various tissues, blood and saliva of sheep in Group 1 killed at 24 hour intervals

Material collected	Animal No. and day killed							
	EU 55 Day 1	EU 56 Day 2	EU 57 Day 3	EU 58 Day 4	EU 50 Day 5	EU 52 Day 6	EU 53 Day 7	EU 54 Day 8
Left m. infrapinatus (stained).....	3·5*	5·0	6·0	6·1	>6·6	5·6	NVD†	(3·5)§
Left m. infrapinatus (unstained).....	2·5	3·2	NVD	3·0	NVD	NVD	NVD	NVD
Right m. extensor carpi radialis.....	2·3	NVD	NVD	NVD	NVD	NVD	NVD	NVD
Left and right m.m. semitendinosus.....	2·2	NVD	NVD	NVD	NVD	NVD	NVD	NVD
Left and right m.m. masseter.....	2·5	NVD	NVD	NVD	NVD	NVD	NVD	NVD
m. diaphragma.....	NVD	NVD	NVD	NVD	NVD	NVD	NVD	NVD
Left axillary lymph gland.....	4·0	3·1	4·8	>3·5	NVD	NVD	NVD	NVD
Left and right parotid salivary glands.....	(2·7) §	NVD	NVD	NVD	NVD	NVD	NVD	NVD
Blood (see Table 2).....	NVD	NVD	NVD	1·5‡	NVD	NVD	NVD	NVD
Saliva.....	NVD	NVD	NVD	2·1‡	NVD	NVD	NVD	NVD
Tongue epithelium.....	NVD	NVD	NVD	NVD	NVD	NVD	NVD	NVD
Interdigital epithelium.....	NVD	NVD	NVD	NVD	NVD	NVD	NVD	NVD
Heart.....	NVD	NVD	NVD	NVD	NVD	NVD	NVD	NVD
Brain.....	NVD	NVD	NVD	NVD	NVD	NVD	NVD	NVD
Spinal cord.....	NVD	NVD	NVD	NVD	NVD	NVD	NVD	NVD

* Corrected titre giving mouse LD₅₀ per gram of tissue. † No virus detected.

‡ Corrected titre giving mouse LD₅₀ per ml of blood/saliva. § Specificity of mortality not confirmed.

It was apparent from the discoloration of the tissues by the dye, that there was, within the first 20 hours after inoculation, a rapid drainage of the inoculum from the infraspinatus muscle into the lymphatic system via the axillary lymph gland. During this period virus was detected in low concentrations in various muscles, although it was not possible to demonstrate virus in the circulating blood at this time.

On succeeding days the concentration of virus appeared to increase, probably as a result of multiplication at the site of inoculation only. At the same time the concentration of virus in the immediately adjacent muscle tissue and the associated lymph gland decreased and was no longer detectable after the 4th day.

The detection of virus in low concentration in the circulation of two of the sheep between the 3rd and 4th days, suggests a spill-over from the primary site of multiplication, where the infectivity titres were 10^6 LD₅₀ per gram of tissue or greater. The demonstration of virus in the saliva of donor EU 58 at the same time is in accordance with previous findings.

The febrile reactions were of a very mild nature and would appear to have little relationship to the occurrence of virus in the circulation (Table 2).

On post mortem examination the muscles and organs did not reveal any macroscopic pathological changes which could be associated with virus multiplication or any of the lesions previously observed in the field.

Clinical observations

The animals of Groups 1 to 5 showed no apparent evidence of disease either immediately after injection or during a further four-month period of observation. Febrile reactions of less than 24 hours duration were recorded among sheep of the group injected intraspinally on the 2nd day after infection. Of the six animals in Group 4 which received the intracerebral injection of starch solution in addition to vaccine intramuscularly, two showed slight nervous symptoms. Since these symptoms were suggestive of cerebral trauma, they were not considered significant.

Immunological response

The serum samples collected at intervals from the experimental animals were assayed for neutralizing antibodies to the homologous strain of virus. These results are given in Tables 3 and 4.

It will be observed that the pre-vaccination serum samples contained no antibodies, thus confirming the susceptibility of the experimental animals.

Within the control group antibodies were detected as early as the 6th day after inoculation. The rise in titre continued until the 14th day and then persisted at the same level until the termination of the experiment four months later.

It is apparent from the detection of antibodies that infection was established by the intraspinal and intramuscular routes but not by the intranasal route of inoculation at the given dosage level. Sheep receiving the vaccine intramuscularly followed by intracerebral trauma appeared to developed distinctly higher neutralizing indices than the control group.

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TABLE 2.—Temperature record and incidence of viraemia in sheep of Group 1

Sheep No.	Days after inoculation															
	1		2		3		4		5		6		7		8	
	‡	6	20	30	46	54	69	77	104	128	152	176				
EU 55	103* NVD	NVD	102.4 NVD													
56	102 NVD	NVD	103.8 NVD	NVD	103.4 NVD											
51	101.6 NVD	NVD	102.6 NVD	NVD	103.8 NVD	NVD	102.2 NVD									
58	102.2 NVD	NVD	102.8 NVD	NVD	102.8 NVD	†(1.6)	101.8 NVD	102.4 †(1.5)								
50	103.4 NVD	NVD	102.8 NVD	NVD	104 NVD	103.6 NVD	102.4 NVD	102.4 NVD	102.4 NVD							
51	103.2 NVD	NVD	103.8 NVD	NVD	103.8 NVD	102.8 NVD	103.8 NVD	103.8 NVD	102.4 NVD	103.6						
53	102.8 NVD	NVD	102.8 NVD	NVD	103.4 NVD	102.4 NVD	102.8 NVD	102.8 NVD	103.2 NVD	103.6 NVD	102.8 NVD					
54	102 NVD	NVD	102.2 NVD	NVD	103.4 NVD	103.6 †(2.8)	102.4 NVD	105.2 †(1.8)	102.4 NVD	102.6 NVD	103.8 NVD	102.8 NVD				

* Degrees Fahrenheit †(1.6) Viraemia detected at a titre of $10^{1.6}$ LD₅₀ per ml of blood.
 NVD No viraemia detected.

TABLE 3.—*Neutralizing antibody response of sheep in Groups 2 and 4*

Group and route of inoculation	Animal No.	Days post vaccination and log N. I of 1:5 serum dilution		
		0	22	105
2—Intranasal.....	Pooled sera	0	0	—
	EU 40	0	NT*	0.2
	41	0	NT	0.2
	42	0	NT	0.2
	43	0	NT	0.0
3—Intraspinal.....	Pooled sera	0	1.0	—
	EU 36	0	NT	2.4
	37	0	NT	2.5
	38	0	NT	1.4
	39	0	NT	1.0
4—Intramuscular with cerebral trauma	Pooled sera	0	2.9	—
	EU 29	0	2.8	3.4
	30	0	3.2	3.0
	31	0	2.2	2.4
	32	0	3.0	3.5
	33	0	1.8	1.0
	34	0	2.0	2.0

* NT not tested

TABLE 4.—*Development of the antibody response in sheep of Group 5*

Group and route of inoculation	Animal No.	Days post vaccination and log N.I. of 1:5 serum						
		0	6	10	14	18	22	131
5—Intramuscular	Pooled sera	0	1.7	1.9	2.1	1.9	1.8	—
	EU 44	—	—	—	—	—	—	2.5
	45	—	—	—	—	—	—	2.75
	46	—	—	—	—	—	—	2.0
	47	—	—	—	—	—	—	1.3
	48	—	—	—	—	—	—	3.0
	49	—	—	—	—	—	—	2.0
	60	—	—	—	—	—	—	2.5
	61	—	—	—	—	—	—	3.0
	63	—	—	—	—	—	—	1.9

DISCUSSION

The results obtained with the 1st group of sheep confirm the observations of previous workers (Mowat, 1961; Burrows, Geering, Mowat & Skinner, 1963) who showed that in cattle some modified strains of virus multiplied in the muscular tissue at the site of inoculation. With this strain of virus in sheep, there was no evidence to suggest multiplication in any of the predilection sites associated with virulent virus, such as the tongue or interdigital epithelium. Furthermore no spread of infection within the inoculated muscle appeared to occur and the muscle surrounding the stained site appeared to be unaffected.

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In an unpublished report on the occurrence of post vaccinal complications following the use of modified RV11 vaccine under field conditions, Tustin, Howell & Basson (1961) described the development of paralysis in a low percentage of vaccinated sheep. Two syndromes were observed which varied with the age of the animal concerned. Young lambs up to the age of three months developed clinical signs from the third day onwards; the shoulder of the inoculated limb dropped slightly, the muscles became stiff and their tonus was increased. Later, the limb was flexed and carried and any attempt to walk produced evidence of acute pain. Within six to twentyfour hours of the onset of these symptoms the remaining limbs of the body became involved and death invariably followed within the ensuing 24 hours. These clinical signs of severe disease in lambs are similar to the muscular involvement seen in young animals as a result of infection with virulent field virus and there is a striking resemblance to the histopathological findings described in mice by Platt (1956).

In older animals the clinical signs were less severe and the onset was not until four to six weeks from the time of vaccination. The onset of paralysis was slower and recovery was protracted. In some cases affected animals would lie for a week or more with the limbs stretched to the side, retaining full sensitivity but completely unable to exercise any motor function. The signs observed in adult sheep in the field were frequently confined to the limb into which the intramuscular injection was given. The histopathological findings were in full agreement with those described by Nobel, Neuman & Rippin (1963) and their demonstration clarified to a certain extent some of the features of the disease which were at first difficult to explain. In those animals which died after a short incubation period severe muscular lesions were evident, whereas in those animals which had experienced long incubation periods the paralysis was essentially due to the presence of an ascending myelitis or encephalomyelitis.

It would seem that, as in the experimental sheep, a high antibody level would be present by the time of onset of symptoms and the dissemination of the virus throughout the body would be restricted. In the present experiments viraemia was only present when the infective titre in the inoculated muscle reached a value above 10^6 LD₅₀.

In the present study none of the field observations has been reproduced. In the field, not all farms experienced the mortality which has been described and, on farms where the syndrome appeared, only 5 per cent of the sheep immunized were affected and these were almost exclusively confined to the 1 to 3-month age group. In the present experiments only 31 sheep were involved and they were all over 15 months old, but a deliberate attempt was made to bring to light any latent neurotropic properties of the strain by inoculating the vaccine intraspinally or intramuscularly with associated intracerebral trauma. On the other hand, it was not possible to reproduce the extremes of environmental conditions under which the affected sheep had been held in the field. Skinner & Knight (1964) have shown that in guinea-pigs the clinical response to infection by intradermal or intramuscular inoculation of modified strains can vary widely between groups, depending on the type and hardness of the floor on which they are maintained. A similar variation in response of sheep under different conditions might account for the difference between the experimental findings and the field observations.

SUMMARY

A duplicate sample of the RV11 vaccine used in the field against the SAT1 epizootic in South West Africa in 1960–1961 retained its infectivity after storage for three years at -20°C . When inoculated intramuscularly into sheep 15 to 18 months old, it stimulated an antibody response. The site of multiplication of the modified virus appeared to be the muscle tissue into which the vaccine was inoculated. A viraemia was detected in a few sheep three to four days after vaccination, but no other clinical signs or macroscopic lesions were observed on post mortem.

Attempts to induce involvement of the central nervous system by simultaneous intracerebral inoculation of starch, or by intranasal or intraspinal inoculation of the vaccine diluted hundredfold, were unsuccessful. No evidence of abnormal behaviour was detected over a 15 week period of observation.

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