**Infection of sheep and monkeys with Langat virus:
cross-protection against other viruses of the
Russian spring-summer complex**

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Langat virus, a member of the Russian spring-summer (RSS) complex of group B arboviruses, was isolated from *Ixodes granulatus* ticks found on forest rats near Kuala Lumpur, Malaya (Smith, 1956). It was shown to be distinct from the other members of the complex by Clarke (1962). Natural infections with Langat virus appear to be rare and no overt disease has been observed: of 386 Malayan human sera (including 215 from forest people) only two (of the latter) were found to have neutralizing antibody. In thirty-two patients with malignant disease, infection with Langat virus (TP-21-7th mouse passage) usually caused only a mild febrile illness although evidence of encephalitis was found in two (Webb, Wetherly-Mein, Smith & McMahon, unpublished). Price, Lee, Gunkel & O'Leary (1961) showed that one strain of Langat virus (TP-21-9) which had been passaged in chick embryo tissue cultures did not produce encephalitis when inoculated intracerebrally into rhesus monkeys and that when three live viruses (yellow fever, West Nile and Langat (TP-21-9)) were given in series there was subsequently good protection against a wide range of group B arboviruses. Price *et al.* (1963*a*) reported that 70 % of the plaque-purified clones prepared from the 12-20th chick embryo tissue culture passages of Langat virus showed increased virulence for mice inoculated intraperitoneally and caused more lesions of the central nervous system in rhesus monkeys inoculated intracerebrally than the parent 8th mouse passage. There was no comparable increase in virulence following passage in hamster kidney cultures. Shah *et al.* (1962) studied Langat virus in mice and guinea-pigs and demonstrated a good measure of protection against some viruses of the RSS complex. Smorodintsev (1963) has carried out preliminary trials in man with live Langat virus as a vaccine against Russian spring-summer encephalitis.

The present experiments were designed to investigate the possibility of using Langat virus as a live vaccine against louping ill in sheep and against the RSS complex of viruses in man.

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MATERIAL AND METHODS

Viruses

- (i) The 6th and 9th unweaned mouse passages of the TP-21 strain of Langat virus.
- (ii) The Absettarov strain of Central European tick-borne encephalitis virus at an unknown mouse passage level obtained from the Walter Reed Army Institute of Research, Washington, D.C.
- (iii) The W 377 CD IV strain of Kyasanur Forest disease virus at the 8th mouse passage obtained from Dr J. S. Porterfield ex Dr M. Theiler.
- (iv) The Moredun strain of louping ill virus (LI).

Neutralization tests

Qualitative tests were done in 3 to 4-week-old mice by the method of Smith & Westgarth (1957). Quantitative tests were similar to the qualitative tests described by Smith *et al.* (1964), except that sera were mixed with equal volumes of four 10-fold dilutions of virus. The log neutralizing index was the difference between the log LD₅₀ of the virus after incubation with test sera and the log LD₅₀ of the virus after incubation with antibody-free serum. The end-points were calculated by the method of Reed & Muench (1938).

Haemagglutinin-inhibition (HI) tests

These were done at room temperature using 8–16 units of antigens prepared from unweaned mouse brain by high-speed centrifugation (Sabin, 1956; Smith *et al.* 1964).

RESULTS

A. LABORATORY EXPERIMENTS IN SHEEP

*Results of infection**Subcutaneous (SC) inoculation*

Forty-five adult sheep and seven lambs aged 1–7 days were given $10^{1.6}$ – $10^{9.6}$ mouse IC LD₅₀ of 9th passage Langat virus. None showed evidence of clinical disease during the following 3–4 weeks and none of ten adults and two lambs tested showed detectable viraemia 1–5, 7 or 9 days after infection; seven were also tested on the 11th day with negative results. No detectable neutralizing antibody was found 21 days after infection (Table 1).

Intracerebral (IC) injection

Ten adult sheep and four lambs were injected IC with $10^{2.3}$ – $10^{8.0}$ mouse IC LD₅₀ of 9th passage virus. Apart from two traumatic deaths among the adults and one among the lambs, no clinical disease was seen during the following 3–4 weeks. Adult sheep which received $10^{6.0}$ – $10^{8.0}$ mouse IC LD₅₀ developed significant levels of neutralizing antibody to Langat virus but not to louping ill virus. The one given $10^{2.3}$ mouse IC LD₅₀ failed to do so. Of the lambs, one

showed a probable antibody response to Langat virus, but two others given 10-fold more virus showed no response (Table 1). All the lambs were 1 day old at the time of infection.

Protection against louping ill virus

A number of sheep vaccinated either SC or IC with doses of Langat virus ranging from $10^{1.6}$ to $10^{6.0}$ mouse IC LD 50 were challenged with louping ill virus either by the method of Edward (1947) or by direct IC inoculation. Eight out of twelve sheep vaccinated SC with at least $10^{2.3}$ mouse IC LD 50 of Langat virus survived SC challenge with louping ill virus followed three days later by 1 ml. of 2 % starch IC.

Table 1. *Antibody responses in sheep following infection with Langat virus and subsequent challenge with louping ill virus*

Langat virus infection			Neutralizing indices			
			21 days after infection with Langat		21 days after challenge with LI	
			Langat	LI	Langat	LI
SC	Adults	1.6-9.6	< 1.0* (25)	< 1.0* (9)	1.4-1.6† (5)	2.0-2.6† (5)
	Lambs	2.0-6.0	< 1.0* (4)	—	1.0-2.2† (4)	1.7-3.6† (4)
IC	Adults	2.3	< 1.0 (1)	—	—	—
		6.0	1.6* (5)	0.7* (5)	2.6* (3)	2.2* (3)
		8.0	2.0* (2)	0.3* (2)	2.0* (2)	—
	Lambs	6.5	1.2 (1)	—	—	—
		7.5	< 1.0* (2)	—	—	—

() Number of sera examined.

* Pooled sera.

† Range of neutralizing indices for individual sera.

Table 2. *Sheep vaccinated with Langat virus and challenged with louping ill virus*

Langat infection		Louping ill challenge	
Route	Dose (log mouse IC LD 50)	Method	Survivors
SC	1.6	LI SC plus starch IC	0/5
	2.3		3/3
	4.6		2/6
	5.9		3/3
IC	2.3	LI virus IC	1/1
	6.0		3/3
	Nil		1/7 (14 %)
SC	5.9	LI virus IC	0/2
IC	6.0		0/1
	Nil		0/1

All the sheep vaccinated by the IC route survived a similar challenge, but no sheep, whether vaccinated SC or IC, withstood IC challenge with louping ill virus (Table 2). Table 1 shows that after challenge, all surviving animals developed

louping ill neutralizing antibody and that the levels of Langat antibody in the same specimens were not significantly different regardless of their level before challenge.

B. FIELD EXPERIMENTS IN SHEEP

As the viruses of Langat and louping ill are closely related serologically and the former is not known to cause naturally occurring disease in either man or sheep, it was felt that it might be used as a potential live virus vaccine. Since it is transmitted only by ticks, the infected sheep used in the field trial were kept on tick-free land for a month after inoculation. Viraemia has not been detected in sheep, and it was considered, therefore, that there was no risk of infecting ticks.

Vaccination

Fifty yearling sheep (hogs) were obtained in March 1961, from a tick-free area and moved to the West of Scotland College of Agriculture at Auchincruive, near Ayr. After preliminary blood sampling, twenty-five were inoculated SC with $10^{3.0}$ mouse IC LD50 of the 6th mouse passage Langat virus and the remainder kept as controls. They were retained there on tick-free pasture for 1 month, then, at the end of April, were placed in equal numbers of vaccinated and control hogs on three hirsels (areas of hill sheep farms confined naturally by burns, ravines, etc.) on Camlarg, Dalcairnie and Knockgray farms in an endemic louping ill area in Ayrshire (see map, Smith *et al.* 1964).

Results of field exposure

Deaths from louping ill

Only those deaths confirmed by virus isolation from the central nervous system were accepted. The development of neutralizing and HI antibody to louping ill was taken as evidence of infection.

Louping ill killed one of six vaccinated hogs at Camlarg. No cause of death was found in the seventh hogg. All of five non-vaccinated hogs on the same hirsle died of louping ill; a sixth had a *Pasteurella* septicaemia and the seventh was lost. At Dalcairnie, two of the four vaccinated hogs infected with louping ill and all three infected in the non-vaccinated group died of louping ill. Thus, among the vaccinated hogs which became infected on both hirsels there were 3/10 deaths from louping ill compared with 8/8 in the infected non-vaccinated hogs (Table 3). As there was no evidence of infection at Knockgray up to the end of June, the hogs there were transferred to Camlarg where, although 93% (13/14) became infected, no evidence of disease was observed.

Antibody responses

None of the sheep had antibody to either virus before vaccination. Twenty-one days after Langat infection eight of twenty-five had Langat neutralizing antibody and three HI antibody. After 13 days exposure on the Camlarg hirsle, antibody responses showed that the majority had been infected with louping ill. All the vaccinated animals had either or both HI and neutralizing antibody to both louping ill and Langat; all except one of the control animals developed similar

antibodies. Those animals which survived retained louping ill neutralizing antibody for the 4 months to the end of the experiment. HI antibody titres, however, fell markedly; the mean Langat titre fell from 1/320 in May, to 1/40 in June and to 1/10 in September, while the corresponding mean louping ill titres were 1/320, 1/80 and 1/20. On the Dalcairnie hirsels no change in antibody was detected after 8–14 days exposure. The majority of the louping ill infections and deaths occurred

Table 3. *Field trial: infection rates and death rates from louping ill*

Hirsels	Number per group exposed	Vaccinated		Non-vaccinated		Total infection rates
		Infected	Deaths from LI among infected	Infected	Deaths from LI among infected	
Camlang	7	7	1/6*	7	5/5†	14/14 (100 %)
Dalcairnie	11	4	2/4	3	3/3	7/22 (32 %)
Total	18	11	3/10	10	8/8	21/36
%	—	—	30	—	100	58
Knockgray	7	0	0	0	0	0/14 (0 %)
Camlang July/Sept.	7	6	0/6	7	0/7	13/14 (93 %)

* One died of another cause.

† One died of *Pasteurella septicaemia* and one was lost.

between the May and June bleedings and only two of the animals infected survived to demonstrate HI and neutralizing antibody in June and September. On the Knockgray hirsels no antibody changes attributable to louping ill infection had occurred by the end of June. After transfer to Camlang all the control and all except one of the vaccinated animals developed louping ill HI and neutralizing antibody.

The infection rate on the Camlang hirsels was 100 % in May and 93 % in July–September. On Dalcairnie it was 32 % in May–June.

C. EXPERIMENTS IN MONKEYS

Intracerebral

Results of infection

Two rhesus monkeys were inoculated IC with $10^{7.0}$ mouse IC LD₅₀ of 6th passage Langat virus. No fever (apart from the first 48 hr.) or other symptoms were observed during 40 days observation. No virus was isolated from their blood on the 7th day or from their brains on the 40th day. Their serology is shown in Table 4.

At autopsy monkey A was found to have extensive generalized tuberculosis. The brains of these monkeys were fixed by perfusion *in situ* and examined by Dr V. Udall of the Wellcome Research Laboratories. He reported that in monkey A there was no evidence of a virus infection in the central nervous system and in monkey B the only lesion observed was a small area of neuronal damage and neuronophagia in the embolic nucleus of the cerebellum. The significance of this finding is not clear.

Subcutaneous

A cynomolgus monkey (C) was given $10^{6.0}$ mouse IC LD₅₀ Langat virus SC. Viraemia was detected in low titre on the 2nd and 4th days by inoculation of unweaned mice but not subsequently up to the 21st day when tested on alternate days. The monkey had no fever or other symptoms. The serology is shown in Table 4.

Table 4. *Homologous antibody responses following Langat virus infection in monkeys*

Route	Intracerebral				Subcutaneous		Oral	
Monkey	A		B		C		D	
Day	HI*	NI†	HI	NI	HI	NI	HI	NI
0	< 10	0.0	< 10	0.0	< 10	0.0	< 10	0.0
4	—	—	—	—	< 10	—	< 10	—
7	< 10	—	< 10	—	—	—	—	—
11	—	—	—	—	40	—	20	—
30	—	—	—	—	—	> 1.8	20	> 1.8
40	20	2.4	80	2.5	—	—	—	—

* HI = Reciprocal of highest dilution causing complete haemagglutinin-inhibition.

† NI = Neutralizing index.

Oral

A cynomolgus monkey (D) was given $10^{8.0}$ mouse IC LD₅₀ of Langat virus orally. Viraemia was detected on the 2nd, 4th and 7th days but not subsequently up to 21 days. The monkey had no fever or other symptoms. The serology is shown in Table 4.

*Protection against related viruses**Central European tick-borne encephalitis*

Monkeys C and D together with two controls (E and F) were given $10^{5.0}$ mouse IC LD₅₀ of the Absettarov strain SC. Three days later 1 ml. of 2% starch was given IC. No symptoms or fever were detected in controls or in C and D. They were killed 22 days after challenge, the brains were fixed by perfusion *in situ*, and were examined histologically by Dr V. Udall, who reported as follows:

Subcutaneously vaccinated monkey (C). Brain. No evidence of encephalitis or of starch inoculation.

Orally vaccinated monkey (D). Brain. No evidence of virus infection but there is a small zone of inflammation probably due to the injection of starch.

Control monkeys (E). Brain. There are many small foci of infiltration and cuffing throughout the cerebral hemispheres. In the cerebellum there is patchy loss of the granular layer and of some Purkinje cells. The fastigial nucleus is damaged. *Cord.* There is infiltration and some chromatolysis of the anterior grey matter.

(F). *Brain.* There is evidence of injection trauma in the forebrain and of meningoencephalitis affecting numerous sites. In general, there is infiltration and cuffing with less effect on the neurones, except in the cerebellar cortex, fastigial nucleus and anterior horn cells of the cord. The inflammatory reaction can be seen

in the anterior commissure, and in the rhinencephalon inferior to it, the thalamus, subthalamic nucleus, substantia nigra and nuclei pontis. There is no evidence of disturbed function in any other tissue.

Thus although symptoms were not produced, there was marked encephalomyelitis in the control monkeys but not in the monkeys previously infected with Langat virus. Twenty-two days after challenge with Absetterov virus, monkey C had shown only a twofold rise in HI antibody to Langat virus, monkey D, a fourfold rise. In contrast, the control monkeys showed increases from $< 1/10$ to $1/160$ and $> 1/1280$.

Kyasanur Forest Disease (K.F.D.)

A similar experiment (summarized in Table 5) was made with K.F.D. virus. The vaccinated monkeys showed no symptoms after K.F.D. infection but the controls were dull and had no appetite from the 4th to the 9th day. Viraemia was observed in the control but not in the vaccinated monkeys. The brains were perfused on the 28th day after challenge and histological examination by Dr V. Udall showed no evidence of encephalomyelitis in the vaccinated monkeys or in one of the controls. The other control had diffuse encephalitis involving the frontal, parietal and hippocampal areas of the cerebral cortex and the dentate nucleus.

Table 5. *Vaccination of monkeys with Langat virus and challenge with Kyasanur Forest disease virus*

Route	Vaccination (Langat)		Challenge (K.F.D.)		
	Dose (log mouse IC LD 50)	Viraemia (days)	Dose (log mouse IC LD 50)	Viraemia (days)	Histology ence- phalitis
Oral	5.8	2-6	6.4	Nil	—
SC	3.8	1-6	6.4	Nil	—
Control	Nil	—	6.4	1-8	+
Control	Nil	—	6.4	1-8	—

DISCUSSION

Langat virus is clearly of very low pathogenicity for sheep; it causes no illness even by intracerebral inoculation, does not cause detectable viraemia and antibody responses are absent or minimal. In contrast, monkeys can be infected by the intracerebral, subcutaneous and oral routes without clinical or histopathological disease but with marked viraemia and strong antibody responses. The 6th mouse passage appears less virulent for monkeys than the 8th which was studied by Price *et al.* (1963a). In view of the known susceptibility of arboviruses to acid, the oral infection presumably took place in the mouth, pharynx or oesophagus.

One third of the hogs vaccinated with Langat virus developed either neutralizing or HI antibody to it before leaving Auchincruive. Infection with louping ill virus stimulated formation of both types of antibody to Langat virus. However, there was no significant difference in the HI titre to either Langat or louping ill

viruses whether the animals had been vaccinated or not. Langat HI titres tended to fall more rapidly than louping ill titres following louping ill infection: Langat titres fell an average of eightfold in the first 6 weeks compared with fourfold for louping ill titres. In the following three months both fell a further fourfold.

Because of the poor antibody responses in sheep, Langat virus makes a relatively poor vaccine in them against RSS complex of viruses. On the other hand it is an effective vaccine in monkeys and probably in man. The virus causes viraemia and good antibody responses in patients with malignant disease and although encephalitis has occurred in these abnormal individuals, they were specially at risk because of malignant deposits in the central nervous system (Webb *et al.* unpublished).

Smith *et al.* (1964) discussed the control of louping ill and the outstanding problems in the pathogenesis of the encephalitis. One of these problems, the influences of age and nutrition on the risk of encephalitis, is posed by the hogs exposed at Knockgray. None of them showed evidence of infection while exposed there but 93 % of them were infected in July–September at Camlarg. None showed evidence of disease although among similar hogs exposed at Camlarg from April to June, 73 % of those infected died of louping ill.

Of sheep vaccinated subcutaneously with at least $10^{2.3}$ mouse IC LD₅₀ of Langat virus and challenged with louping ill virus by the method described by Edward (1947) 67 % survived; this is about the same as the survival rate in laboratory sheep vaccinated with formalinized sheep brain vaccine and challenged in the same way (O'Reilly & White, unpublished). Among sheep infected with louping ill in the field trial 30 % of the Langat-vaccinated hogs and 100 % of the non-vaccinated controls died of louping ill. Although this difference is significant, the protection is not so much better than the formalinized sheep brain vaccine as to justify the use of a live virus vaccine.

Langat virus infection protects monkeys against encephalitis and reduces or eliminates viraemia in them due to other viruses in the RSS group. This confirms work by Price *et al.* (1963*b*) who followed us in using Edward's method of challenge employing peripheral inoculation followed by starch intracerebrally. The possible use of Langat virus as a vaccine in man shows promise, either alone against the RSS complex of viruses (Smorodintsev, 1963), or in combination with other group B arboviruses against the whole group (Price *et al.* 1961).

SUMMARY

1. The susceptibility of sheep to infection with Langat virus has been studied. No viraemia or symptoms were detected in sheep inoculated either subcutaneously or intracerebrally.
2. Only those sheep inoculated intracerebrally with $10^{6.0}$ – $10^{8.0}$ mouse IC LD₅₀ of virus developed significant quantities of neutralizing antibody.
3. Two-thirds of sheep vaccinated with varying doses of Langat virus withstood subcutaneous challenge of louping-ill virus followed by intracerebral starch. All the intracerebrally vaccinated sheep survived this form of challenge but no sheep,

whether vaccinated subcutaneously or intracerebrally, withstood intracerebral challenge of louping ill.

4. In a field trial, three of ten hogs vaccinated with Langat virus and exposed to natural louping ill infection at Camlarg and Dalcairnie died of the disease compared with all eight of the non-vaccinated hogs. At Knockgray, there was no louping ill infection, but 93 % of the hogs from this hirsle developed louping ill antibody after transfer to Camlarg.

5. Monkeys infected intracerebrally, subcutaneously or orally with Langat virus showed a low titre viraemia without clinical symptoms or histological changes in the brain and developed high titres of antibody. Vaccinated monkeys challenged with either Central European tick-borne encephalitis or Kyasanur Forest disease viruses remained healthy compared with control monkeys which showed evidence of disease.

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