EXPERIMENTS IN CONNEXION WITH AN ATTEMPT TO PRODUCE A NEUROTROPIC STRAIN OF VACCINIA VIRUS IN SHEEP

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CONTENTS

The experiments now described were carried out in Lagos and Khartoum, and although the hoped-for end-result, the production of a sheep strain of neurovaccinia, was not realized, certain points of interest have arisen and are recorded in the hope that they may be of help to other workers concerned with vaccinia vaccine production.

Experiments in Lagos

Owing to the difficulty of obtaining calves in Lagos, vaccinia vaccine is produced from sheep. The original seed vaccine, which was used for initiation purposes, was obtained from the Government Research Institute at Colindale through the kindness of Col. W. D. H. Stephenson. It was labelled 'No. 708 calf strain'. This seed vaccine gave good results when used at Vom in calves obtained from the Veterinary Department. Vom is situated in the plateau area of Northern Nigeria. Repeated passages were then made in sheep, and by combining a careful choice of animals with selection of the results a strain of vaccinia adapted to sheep was eventually obtained from which all subsequent strains used in Lagos have been derived. This sheep strain was evolved by Mr R. Bowrey, Laboratory Superintendent.

Sheep strain No. 49 was chosen for use in the experiments which form the subject of this paper. This strain had been stored in glycerine (dilution 1/3) at 0° C. for 12 months, and both anaerobic and aerobic cultures made from it were negative. Potency tests in rabbits, using standard dilutions, were satisfactory.

Initial attempts to infect sheep by the intracranial method with this strain proved abortive and neuro-adaptation in other animals was therefore undertaken with a view to subsequent sheep passage. Ether anaesthesia was

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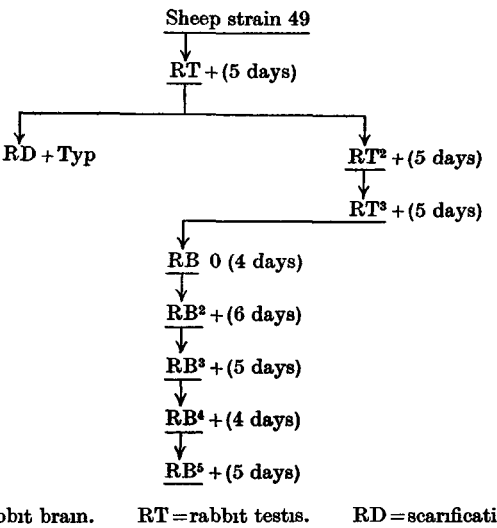
employed in all experiments, except where rabbits were used. Both intratesticular and intracerebral inoculations can be carried out in these animals without causing any noticeable discomfort. Routine control cultures on blood agar and in broth were made throughout and were negative unless stated to the contrary.

A. *Rabbits*

Levaditi (1923) recommends initial intratesticular passages prior to attempting the neurotropic adaptation of vaccinia virus. Accordingly, sheep strain No. 49 was passaged intratesticularly in rabbits. The technique followed that recommended by Noguchi (1915). The details of these primary testicular passages with subsequent neurotropic adaptation is shown in Table 1.

Table 1. *Rabbits*



RB = rabbit brain. RT = rabbit testis. RD = scarification test.

A successful result was obtained with the 1st passage. The testes were found to be swollen and deeply congested on the 5th day, and at the 3rd passage an intensely haemorrhagic reaction was obtained.

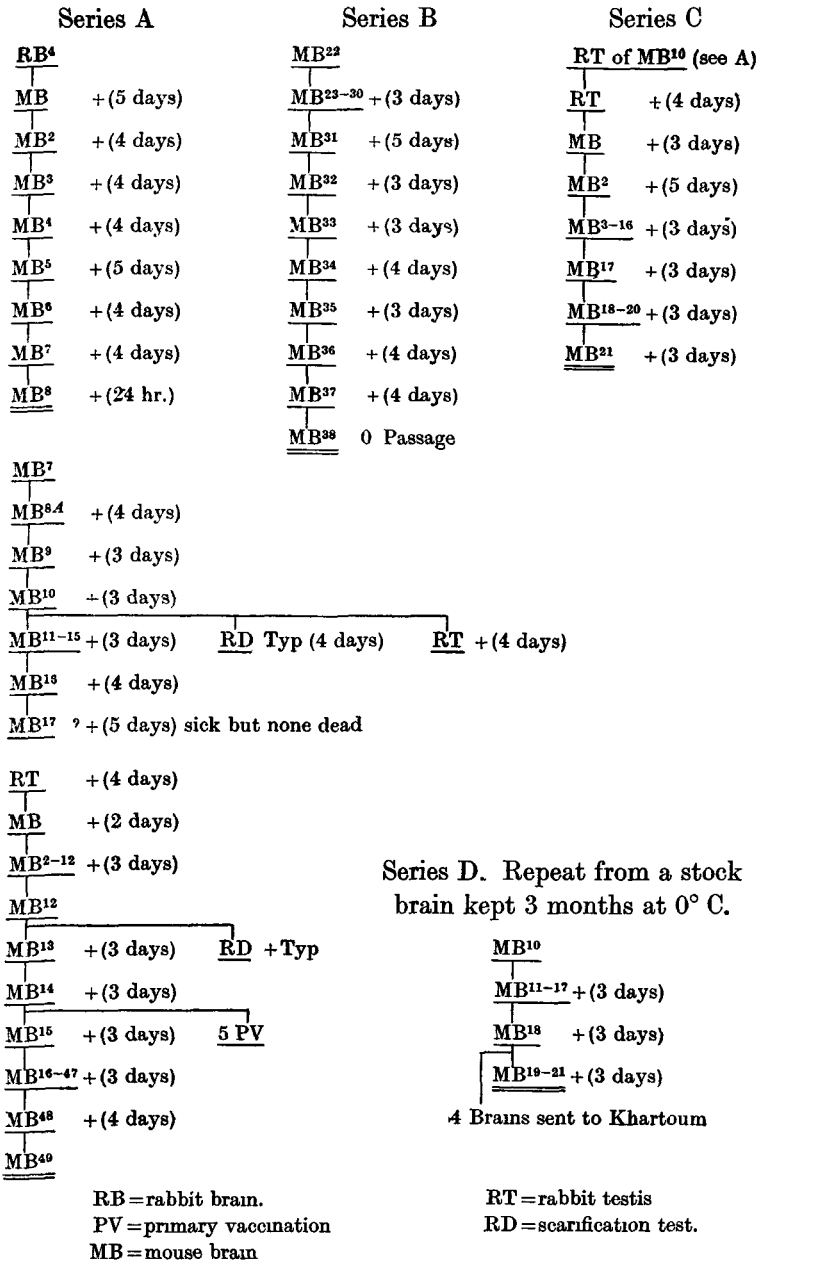
In the cerebral experiments 0·25 c.c. of a 1/10 emulsion of testis material in saline, lightly centrifuged to throw down coarse particles, was used for the 1st passage. Thereafter brain tissue was used in the same dilution.

B. *Mice*

The mice employed were all derived from the Swiss strain susceptible to the virus of yellow fever. Four to six mice were used for each passage and only young animals (6–8 weeks) were used in the experiments under discussion. Older stock (6–8 months) were investigated later, and it was found that though they could be in most cases readily infected with the virus the incubation period was prolonged. For the inoculum 0·03 c.c. of a centrifuged 1/10 dilution

in saline was employed throughout. Death usually occurred suddenly, with or without premonitory symptoms, on or about the 3rd day. Some of the mice exhibited hypersensitivity and varying degrees of spastic paralysis. The experiments are detailed in Table 2.

Table 2. *Mouse passage*



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It will be seen that the 1st mouse passage (series A), made with rabbit-brain material, was successful, and further passages were made without difficulty until the 8th was reached when the mice died within 24 hr. Contamination was suspected (cultures were subsequently found to be negative) and a reserve brain from the previous passage, which had been stored in 50% glycerine at 0° C., was used to continue the series. That the virus in question was in fact vaccinia is proved by the results of the dermal and testicular inoculations in rabbits.

The virulence of the virus seems to have decreased at the 17th passage, since symptoms were not noted until the 5th day and no deaths had occurred. A testicular (rabbit) passage was made in order to rejuvenate the strain and forty-eight further passages were made. The 49th passage was negative. In this series the virus was again controlled by a dermal test in a rabbit and by five primary vaccinations in babies.

A second series (B) was begun, using a 22nd passage mouse brain which had been stored in 50% glycerine at 0° C. for a period of 3 months. Passages were successful until the 38th, when the virus again appeared to have lost virulence.

A third series (C) was then initiated, using the rabbit-testis passage of a 10th mouse-brain passage (series A). A testis (rabbit) passage was first made and this was followed by twenty-one successful mouse-brain passages when the series had to be interrupted owing to shortage of suitable mice. Seven primary vaccinations in babies were made with material from the 17th passage of this series and also an intradermal potency test in a rabbit. The results of the latter were:

Dilutions ...	1/10,000	1/50,000	1/100,000	1/200,000
Results	+ + + +	+ + +	+ +	+

\+ + + + Inflamed raised indurated area approximately 1½ in. diameter. Haemorrhagic centre

\+ Inflamed papule

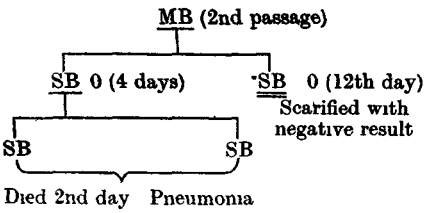
The primary vaccinations in babies (series A and C) and the rabbit scarification tests (series A) gave excellent results with well-formed vesicles without induration or haemorrhage. Series D is given merely to show the source of the virus which was used in the experiments carried out in Khartoum.

C. *Sheep*

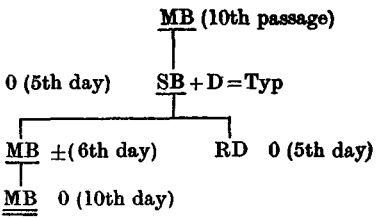
The sheep used in these experiments were obtained from Kano (Northern Nigeria). It was difficult to assess their age, but 2 years may be taken as an approximate average. The inoculum used for intracerebral passage was 0·5 c.c. of 1/10 saline emulsion (lightly centrifuged) of brain or testicular material. The experiments are shown in Table 3 and consist of three series, in all of which attempts were made to infect sheep with vaccinia virus intracerebrally, using mouse brain and rabbit testis as the inoculum. Mouse-brain tissue was used in series A and B with unsuccessful results in both. In series B the mouse-brain virus was inoculated (scarified) upon one flank and groin of the

Table 3. *Sheep and rams*

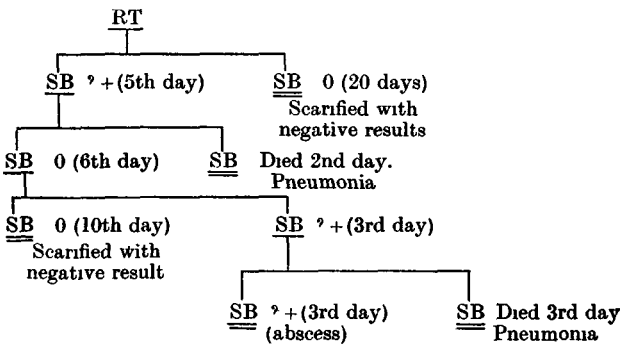
Series A



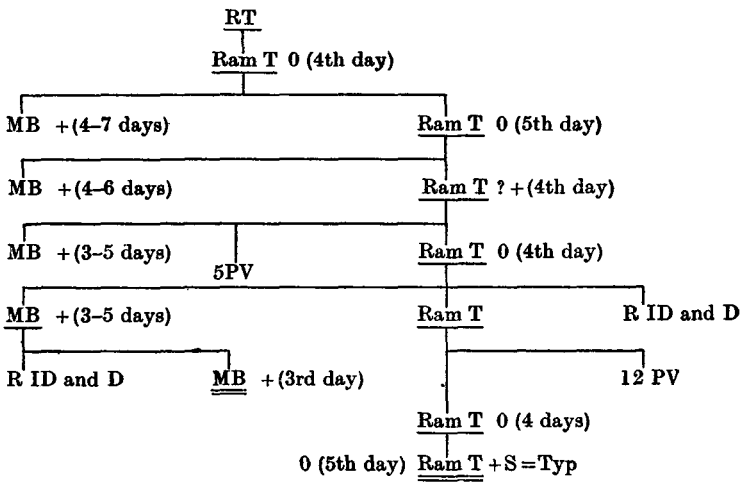
Series B



Series C



Series D



MB = mouse brain.
RT = rabbit testis.
Ram T = ram testis.
SB = sheep brain.
D = scarification.
ID = intradermal.
PV = primary vaccinations.
RD = scarification test.

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animal in the hope that a dermal infection might tend to augment the effect of the intracranial passage; the result was again negative except for the excellent dermal reaction which incidentally proved that the sheep was non-immune and that the virus was potent.

In series C, testicular tissue (rabbit) was used as the source of virus. It is shown that in the 1st passage symptoms were noted at 5 days. These consisted of loss of appetite, some spasticity in the hind-limbs and general weakness. Further passages, however, were negative. One animal of the 3rd passage did show symptoms of weakness and general instability, but an abscess developed at the site of inoculation in the next passage (*Staph. aureus* isolated in pure culture) and no further passages were made.

D. *Rams*

In the testicular passage in three animals, shown in detail in Table 3, series D, 2–3 c.c. of a 1/5 emulsion of material in saline was used for each testis. Seven passages were made in series and in only one (3rd) was any alteration noticed in the naked-eye appearance of the organs. In this instance there was a bilateral effusion of clear fluid into the tunica vaginalis with slight flushing of the testes.

In the 6th and 7th passages the animals were also scarified in the groin and one flank with a known potent strain of sheep vaccinia. This scarification was made for the same reason as in series B.

Good takes were obtained in both animals.

Intradermal and scarification tests were made in rabbits using (*a*) material from the 4th testicular passage, and (*b*) the same material after one mouse-brain passage. The results are as follows:

	Rabbit testis control	First mouse-brain passage of ram No. 7	Ram testis 4th passage
Scarification test: Dilution 1/10	Excellent	Excellent	A few small vesicles
Intradermal tests: Dilution 1/100	Not done	+ +	+
„ 1/10,000	+ + + Haemorrhagic centre	+	0
„ 1/50,000	+ +	0	0
„ 1/100,000	+	0	0
„ 1/200,000	±	0	0

Primary vaccinations in babies were made with material from the 3rd and 5th ram testes passage. In the first group (five babies) four excellent results were obtained, one was negative. In the second group (twelve babies) only three reactions of poor quality were noted.

Experimental intracerebral inoculations of the mouse-brain virus were also made in guinea-pigs, Syrian hamsters and 2-year-old bulls, zebu type (four animals), with completely negative results.

Discussion

The facility with which the dermotropic vaccinia virus (sheep strain 49) established itself in rabbit testicular tissue is noteworthy, as also its subsequent rapid adaptation to rabbit-brain tissue as was shown by the occurrence of spastic paralysis (except in the 1st passage) in from 4 to 6 days after inoculation. These results differ from those described by Levaditi (1923) in the initiation of his strain of neurovaccinia. Further, Levaditi was not successful in his attempts to infect mice with neurovaccinia, and it seems that this is the first time in which adaptation of vaccinia virus to mouse-brain tissue has been recorded.

A feature of interest in the sheep experiments is the failure of vaccinia virus to survive for long in sheep-brain tissue as was shown by (*a*) the impossibility of recovering the virus by mouse passage after only one intracerebral passage in sheep, and (*b*) scarification of a rabbit (Table 3, series B) with negative results. This short survival period (less than 5 days) contrasts with its relative longevity in the testes of the ram. It is to be noted that solid immunity to vaccinia virus was conferred upon sheep by intracerebral inoculations (series A, one animal, and series C, two animals). Somewhat similar results, i.e. solid immunity following intracerebral inoculation without exhibition of symptoms, has been noted by Levaditi (1938).

As already stated vaccinia virus was capable of prolonged survival in the testicular tissue of rams. In Table 3, series D, intracerebral inoculations in mice, made with testicular material of the 4th passage (17 days), were positive. That the virus had lost potency, however, was shown by the intradermal and scarification tests in rabbits and in the results of the primary vaccinations.

The absence in general of haemorrhagic or confluent reactions in the skin usually associated with neurovaccinia would seem to distinguish the mouse strain of neurovaccinia from those hitherto recorded. This tendency to severe dermal reactions has been responsible in part for condemning the vaccine for more general use.

Summary

1 It has not been possible to effect the neurotropic adaptation of dermal vaccinia virus (local sheep strain) in sheep.

2. The local strain of vaccinia virus (sheep) has been adapted to mouse- and rabbit-brain tissue.

3. Neurovaccinia (mouse strain) is of high potency and has retained its dermotropic affinities.

4. Mouse neurovaccinia produces typical dermal reactions by the scarification method. Confluent vesicles and haemorrhagic lesions are not usual.

5. Vaccinia virus (mouse strain) is unable to survive for long in sheep-brain tissue but is capable of conferring a solid immunity when inoculated intracerebrally. In rams it can survive for a long period in the testicular tissue.

Experiments in Khartoum

Four mouse brains (in glycerine) with neurotropic vaccinia were received from Lagos (see Table 2, series D).

Experiment I

Two of the brains were ground in 3 c.c. of distilled water, and used for inoculating four mice intracerebrally (0·03 c.c. each) to keep the strain going in mice, one rabbit intracerebrally (0·25 c.c.), two young sheep (about 6 months old) intracerebrally (0·30 c.c.) and titrated by scarification on two rabbits.

(1) *Mice.*

Each of four white mice (Swiss strain) was inoculated intracerebrally with 0·03 c.c. Early in the morning of the 3rd day, it was observed that three of the mice had become hyper-excitable. At about 10 a.m. one of them died, and the other two became depressed and could draw up their hind-legs only with difficulty. These two were killed, their brains were removed under aseptic conditions and kept in glycerine in the refrigerator (4° C.). The fourth mouse kept quite healthy up to the 7th day, when it became hyper-excitable and kept dashing about in the cage with its neck bent to the left; it remained in almost the same condition until it died on the 15th day.

(2) *Rabbits.*

A. *Intracerebral route.* One rabbit was inoculated intracerebrally with 0·25 c.c. On the 3rd day its coat became rough and it looked slightly ill. On the 4th day it appeared better, and subsequently remained healthy. A month later it was tested for the presence of immunity: its belly was shaved, scarified lightly and vaccine lymph of high potency was rubbed in. On the 3rd day after scarification, a few small abortive vesicles developed, which dried up about the 5th day.

B. *Skin scarification.* Potency tests to determine the end-point were carried out on two rabbits by scarification. The technique always used in Khartoum is that employed at the Government Lymph Institute, Colindale (0·1 c.c. of each dilution rubbed over an area of 14·4 sq. cm.). The results read on the 5th day were as follows:

Dilution	1/100	1/1000	1/10,000	1/100,000
1st rabbit	Confluent	Semi-confluent	6 vesicles	2 vesicles
2nd rabbit	Confluent	Confluent	Semi-confluent	7 vesicles

Semi-confluent = more than 12 vesicles.

(3) *Sheep.*

Two young sheep (about 6 months old) were inoculated intracerebrally with 0·3 c.c. of the mouse-brain suspension. Young sheep were used in all the experiments in the hope that they would be more susceptible. On the 3rd day after inoculation, one of them (brown) became shaky and looked definitely

ill but was not paralysed. The other one (white) remained healthy. On the 4th day the brown sheep became better and both of them subsequently kept quite well. A month later they were tested for immunity. Their bellies were shaved, lineal incisions (about 3 in. long and 1 in. apart) were made over the whole of their bellies and vaccine lymph of high potency was rubbed in. The results were read on the 5th day; the brown sheep was completely negative except for two small abortive vesicles, but the white sheep showed a good even take-all over the belly.

Summary

1. A strain of neurovaccinia (Smith) fixed in mouse brains shows an incubation period of 3 days for mice.

2. It exhibited high potency when tested by scarification on rabbits' skin.

3. It did not cause death or produce paralysis in two sheep and a rabbit when inoculated intracerebrally. In the rabbit and one sheep which showed signs of illness a few days after inoculation, a strong immunity to vaccinia was produced.

Experiment II

The two mouse brains of Exp. I were ground in 3 c.c. of distilled water, and the suspension used for inoculating three mice, one rabbit and one young sheep (6 months old).

(1) *Mice.*

Three mice were each inoculated intracerebrally with 0·03 c.c. of the mouse-brain suspension. They became moribund on the 3rd day, and were killed. One of the brains was ground up in distilled water and the suspension used for inoculating four mice intracerebrally. On the 3rd day after inoculation these four mice became moribund; two of them were killed, and their brains removed and stored in glycerine at 4° C. as stock virus. The other two were left to see how they would behave, and died later on the same day.

(2) *Rabbits.*

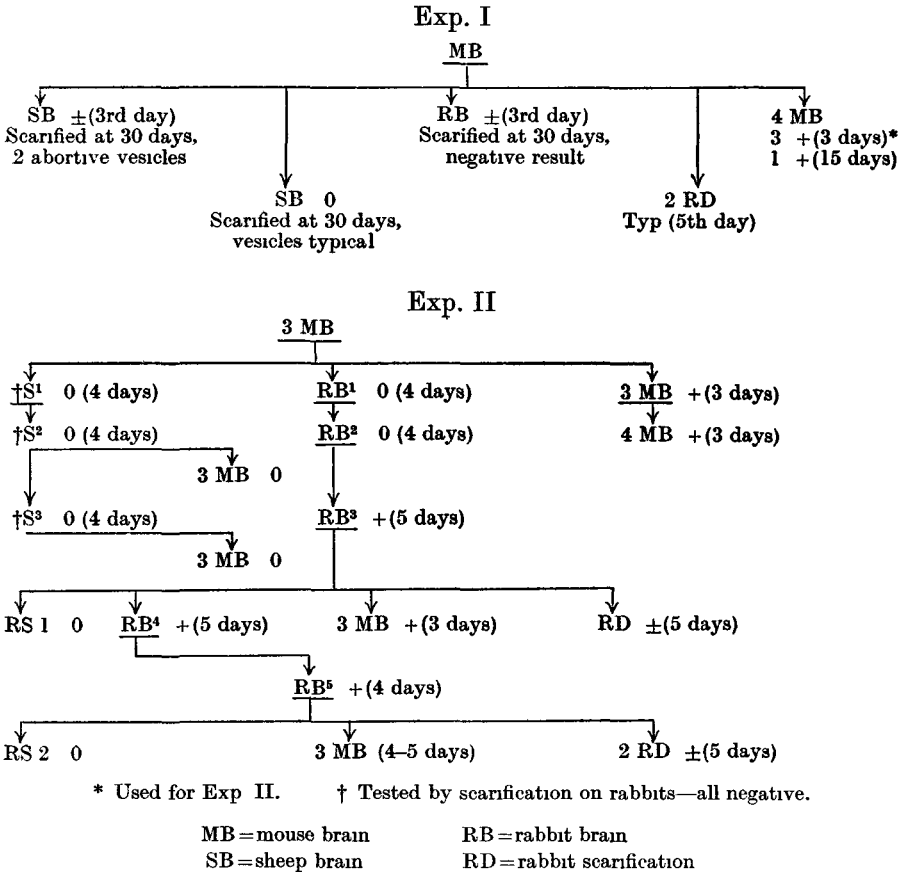
One rabbit was inoculated intracerebrally with 0·25 c.c. of mouse-brain suspension; it did not show any sign of disease when it was killed on the 4th day, and its brain was used for inoculating rabbit No. 2, which also showed no signs of disease. It was killed on the 4th day and its brain passaged to rabbit No. 3. Rabbit No. 3 showed no signs of disease on the 4th day, but early in the morning of the 5th it was found completely paralysed. It was killed and the brain removed and used for inoculating rabbit No. 4, one young sheep (R.S. 1) and three mice, and also titrated for potency on the skin of a rabbit. Rabbit No. 4 was completely paralysed on the 5th day; its brain was passaged to rabbit No. 5. The three mice inoculated with the brain of rabbit No. 3 died on the 3rd day. The potency test was read on the 5th day and showed six well-developed vaccinia vesicles in a dilution of 1/100; all the higher dilutions were negative.

Rabbit No. 5 became completely paralysed on the 4th day and was killed. A suspension of its brain was used to inoculate one young sheep (R.S. 2) and three mice, and tested for potency on two rabbits.

Neither sheep (R S. 1 and R.S. 2), though kept under observation for a month, developed any symptoms. Of the three mice inoculated from rabbit No. 5 one died on the 4th day and two on the 5th day. The results of potency tests for rabbit No. 5 brain when read on the 5th day after scarification were as follows:

Dilution	1/100	1/1000	1/10,000	1/100,000
1st rabbit	3 vesicles	Nil	Nil	Nil
2nd rabbit	4 vesicles	Nil	Nil	Nil

Table 4. *Khartoum experiments*



* Used for Exp II. † Tested by scarification on rabbits—all negative.

MB = mouse brain RB = rabbit brain
SB = sheep brain RD = rabbit scarification

(3) *Sheep.*

One young sheep (No. 1), of about 6 months old, was inoculated intracerebrally with 0·3 c.c. of mouse-brain suspension. It showed no signs of disease and was killed on the 4th day, and its brain passaged to another

young sheep (No. 2). This did not show signs of disease, was killed on the 4th day and its brain passaged to a third young sheep (No. 3). No. 3 kept quite healthy up to the 4th day when it was killed and its brain removed, and stored in glycerine at 4° C. in case it was decided to carry out further passages. Potency tests were carried out by scarification on rabbits' skin, on the brains of Nos. 1, 2 and 3. The results read on the 5th day were as follows:

Dilution	1/10	1/100	1/1000	1/10,000
No. 1	Not done	Nil	1 vesicle	Nil
No. 2	Not done	Nil	Nil	Nil
No. 3	Nil	Nil	Nil	Nil

Groups each of three mice were inoculated intracerebrally with the brains of sheep Nos. 2 and 3: none showed any symptoms and have remained healthy.

Further attempts to fix the virus in sheep by serial passage were discontinued for reasons of expense.

Discussion

Continuous passage of the neurovaccinia in mice showed a regular incubation period of 3 days; if the mice were not killed on the 3rd day, they usually died suddenly.

The findings of Exp. I showed that this strain of neurovaccinia did not at first cause death or produce paralysis in rabbits when inoculated intracerebrally. However, by serial passage, rabbit No. 3 became paralysed on the 5th day after inoculation; as also did No. 4. No. 5 became paralysed on the 4th day. It seems therefore that the strain became fixed for rabbits with an incubation period of 4–5 days, which is in accordance with Levaditi's observation on the behaviour of neurovaccinia in rabbits.

Although this rabbit fixed virus still retained its virulence for mice, the evidence as judged from the slightly longer incubation period for mice between the 3rd and 5th passages suggests that this virulence was lessening. Further experiments in this direction were however not carried out. It may be also noted that this rabbit neurovaccinia remained of low potency when tested by scarification on rabbits' skin.

Three passages in sheep failed to maintain the strain in this animal, and, judged by the completely negative potency tests, it seems that after the 1st passage the virus was unable to survive in sheep brain.

An attempt to infect sheep was then made by using the rabbit fixed strain. One sheep was inoculated with rabbit brain No. 4 and another with No. 5; neither showed any symptoms and both remained healthy for the month during which they were kept under observation. When tested later with a standard vaccine lymph both gave typical reactions.

Summary

1. A strain of neurovaccinia fixed in mouse brains (Smith) showed an incubation period of 3 days for mice.

2. This strain was fixed for rabbits after a few passages with an incubation period from 4 to 5 days.

3. Three serial passages failed to fix the strain for sheep.

4. Rabbit-sheep passage failed also to infect sheep.

General conclusions

An analysis of our combined results shows a substantial measure of agreement. The main conclusions are:

1. A mouse neurovaccinia has been produced in Lagos which is of high virulence for the mouse and rabbit, with an incubation period of 3 days for the former and about 4 days for the latter, following intracerebral inoculation.

2. It was found impossible to adapt this strain to sheep brain, in which tissue it apparently even failed to survive after the 1st passage.

3. It was capable however of some multiplication in the 1st passage as shown by the subsequent acquisition by the animal of strong or complete immunity to vaccinia and by the appearance of transient symptoms in some of the animals.

4. This mouse neurovaccinia retains full dermotropic properties and produces typical vesicles on both rabbits' and babies' skins (Lagos). It does not exhibit the virulent inflammatory and haemorrhagic reactions commonly associated with the usual neurovaccinia of rabbit origin.

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