THE TRANSMISSION OF NEUROTROPIC YELLOW FEVER VIRUS BY STEGOMYIA MOSQUITOES*

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Studies on transmission of neurotropic yellow fever virus by mosquitoes have a bearing on the practical problem of vaccination against yellow fever by the method of Sawyer, Kitchen, and Lloyd (1), and on the more theoretical question of reversion of neurotropic virus to the viscerotropic type.

Dinger (2) has reported the production of typical visceral lesions in monkeys by mosquitoes which carried a strain of neurotropic virus. The insects had been induced to feed on a mixture of infected mouse brains and normal blood. However, the strain of virus had been adapted to mice quite recently (tenth to twelfth passages). We are convinced that the longer established French strain (Theiler (3)) acts somewhat differently in mosquitoes.

The present method of vaccination (1) calls for the injection of a small dose of living neurotropic virus, and for the simultaneous administration of large amounts of immune serum. Considering the small dosage of virus used and the difficulty with which mosquitoes acquire infectivity with the strain, it is inconceivable that vaccinated persons might become a menace to their fellows even in the absence of isolation and protection by screens.

On Nov. 22, 1931, F.L.S. was vaccinated by the usual procedure. 15 hours later he was fed upon by more than 150 stegomyia mosquitoes (Lot 669). On Dec. 22, 1931, *M. rhesus* B10 was inoculated intracerebrally with 1 cc. of foreign serum (as a mild irritant) and was fed upon by the mosquitoes immediately after-

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TABLE I

Miscellaneous Attempts to Infect Mosquitoes with Neurotropic Yellow Fever Virus

Mosquito lots	Source of blood meal	Virus verified in serum of source animal	Virus verified in freshly fed mosquitoes	Later tests in monkeys					Later tests in mice				Observations
				Interval	Bites or injection	Monkey No.	Fever in animal	Immunity produced	Interval	Mouse group	Route of injection	Infection produced	
				days					*days*				
620	*M. rhesus* A1	+	*	14	Bites	A2	+	−	14	R160–165	Ic.	−	Source animal was second intracerebral passage mouse virus in monkeys
624	*M. rhesus* A3	+	*	*	*	*	*	*	1	R180–187	Ic.	−	Source animal fifth intracerebral passage in monkeys
625	*M. rhesus* A3	+	*	*	*	*	*	*	2	R196–199	Ic.	−	Source animal fifth intracerebral passage in monkeys
627	*M. rhesus* A3	+	*	42	Bites	A5	−	−	48	R382–383	Ic.	?	Source animal fifth intracerebral passage in monkeys
631, 632, 633, 634	*M. rhesus* A4	+	*	74	Bites	B3	−	−	41	R381	Subcut.	−	Source animal injected intracerebrally with filtered suspension infected mouse brains
642	Mouse Groups T953–956	*	+	18	Bites	A8	−	−	18	R385	Subcut.	?	Source groups received usual amounts of brain virus and normal serum ip. given in protection tests
648	Artificial mixture brain virus-defibrinated blood	+	+	22	Bites	A6	+	+	22	R424	Subcut.	†	Test Monkey A6 developed clinical encephalitis. Died 10 days after last fever

656	*M. rhesus* A7	+	*	36	Bites	B2	−	−	69	R760	Ic.	†	Source animal belonged to intraspinal neurotropic series
659, 660	Mouse Groups R433, 434	+	+	34	Bites	B7	−	−	60	R761	Ic.	†	Mosquito lots fed on mice at 5 hrs. and at 22 hrs., after large ip. injections of virus
675, 676	Mouse Groups R547, 548	+	+	14	Bites	B5	−	−	14	R629–637	Ic.	?	Lot 675 fed on mice 2 hrs. after ip. injection. Emulsion at once of this lot killed mice in dilution 1:10,000 based on amount of blood ingested
678, 679	*M. rhesus* B1	+	?	14 44	Bites Injection	B9 C9	− +	− +	44	R791	Ic.	+	Source animal inoculated with large amounts of mouse brain virus on 2 successive days. Mosquitoes fed 24 hrs. after last injection
684	Mouse Groups R605, 606	+	+	29	Injection	C3	−	†	15 29	E267 R761	Ic. Ic.	− †	Mosquitoes containing fresh blood of killed mice, in dilutions of 1:100 and probably higher. First subsequent mouse test made with approximately 2 per cent suspension filtered
695	Mouse Groups R718, 719	+	+	37	Bites	D3	+	+	2 37	6 groups R867	Ic. Ic.	? +	Dilutions of mosquitoes on day of infective blood meal, based on amount of blood ingested, killed up to dilution 1:2,500. Some animals died in tests 2 days later. Virus probably present, but not satisfactorily proved. Proved after 37 days

+ = positive. − = negative. ? = doubtful; or infection not satisfactorily proved. Ip. = intraperitoneally. Ic. = intracerebrally. Subcut. = subcutaneously; subcutaneous injections were made into suckling mice.

* No test made.

† Animals died from secondary infections.

ward. The same insects fed upon this animal on Dec. 26, 29, and on Jan. 2, 1932. No temperature reaction developed, and the monkey's serum later proved to have no protective power against yellow fever virus in mice.

In Oct., 1930, two mosquito transmission experiments were performed, one with Asibi strain virus adapted to mice (22nd passage), and one with French strain virus (100th passage in mice). In each experiment a heavy suspension of infected mouse brains was injected into a monkey (*M. rhesus* S1 and *M. rhesus* S2); both animals showed fever on the next day; and mosquitoes were allowed to feed at approximately 24 hours following the inoculation of virus. Neither monkey died of yellow fever. After suitable incubation periods the mosquito lots were permitted to feed on normal monkeys, and the latter subsequently developed fever. The temperature of the animal (*M. rhesus* S3) which received Asibi strain virus through mosquitoes reached 105.4°F. on the 5th day; the temperature of the one (*M. rhesus* S4) which received French strain virus reached a maximum of 104.3°F. on the 14th day after exposure to mosquito bites. Both animals were afterward given test doses of virus and were found to be immune; preceding the immunity test, the serum of *M. rhesus* S4 (which had received French strain virus) gave a strongly positive complement fixation reaction with yellow fever liver antigen.

The experiments listed in Table I were performed for the most part during the last quarter of 1931 and early in 1932. In every case the French neurotropic strain of virus was employed, in passages from the 149th to the 181st. These experiments represent by no means all the attempts made to transmit the neurotropic virus through mosquitoes.

Some difficulty was experienced in determining the most favorable period after the inoculation of virus for the mosquitoes to feed. Monkeys inoculated by the subcutaneous or by the intraperitoneal route with infected mouse brains do not invariably develop fever. Animals which do become febrile following inoculation by these routes, or by either the intracerebral or the intraspinal route as well, do not always have virus in the blood stream at the time of fever. Consequently at first many mosquito lots had to be discarded because the blood of the host was non-infective at the time of feeding. In later experiments mosquitoes were allowed to feed on monkeys at 24 to 48 hours after intraperitoneal or subcutaneous inoculation, and on mice at 2 to 24 hours after intraperitoneal inoculation of virus.

Mosquitoes did not feed well either on artificial mixtures of infected mouse brains and normal defibrinated blood, or on infectious blood *in vitro*, with or without the addition of glucose. Several methods were tried of attracting the insects and inducing them to feed, but none of them ever became fully engorged.

In the single experiment included in Table I in which artificial feeding was employed, the insects of Lot 648 fed on an infected brain-normal blood mixture through the shaven and scraped skin of a recently killed guinea pig. During the time of feeding the lot was divided; some of the insects were kept at incubator temperature (37°C.), and the others were left at room temperature (27°C.). At neither temperature was feeding satisfactory, but in the cage kept at 27°C. more mosquitoes were found afterwards with visible blood in their abdomens.

From the combined lot thirteen insects, which contained recently ingested infective material, were ground up in 3 cc. of 10 per cent normal monkey serum; of this suspension, 0.25 cc. was injected subcutaneously into each of six suckling mice. Three of the latter died within 48 hours; one was dead and two were sick on the 6th day. Transfer of brain material from the sick animals brought down the subinoculated groups with yellow fever on the 4th day. 22 days later (Nov. 11) the remaining mosquitoes in Lot 648 were allowed to feed on *M. rhesus* A6. On Nov. 18 and 19 the monkey had a slight fever. On Nov. 23 his temperature suddenly rose to 106°F., and it remained above 105°F. during Nov. 24 and 25. On Nov. 26 the temperature dropped to 100°F. The animal was discovered lying down—weak, but excitable. There was present marked tremor of arms and head, occasional nystagmus, well developed wrist drop, some muscular incoordination, and difficulty in walking. These signs remained salient for 4 days, and never entirely disappeared. Blood taken 7 days after the last fever gave perfect protection against virus in mice. The temperature of the animal continued almost constantly subnormal, and weakness became progressively more marked. On the 10th day after the last fever the monkey was killed when moribund.

As judged by the symptomatology of *rhesus* monkeys inoculated intracerebrally with neurotropic virus (4), the syndrome presented by this monkey immediately after fever was that of acute encephalitis. It was conjectured that the virus localized in the brain because of irritation resulting from an old depressed fracture in the right frontal region of the cranium. However, this old injury may have played no part in the etiology of the acute infection. At the time of death encephalitis had somewhat subsided clinically. Nevertheless upon microscopical examination a few vessels with cuffing were found in Ammon's horn and in the brain stem; necrotic ganglion cells were noted in the medulla oblongata.

It seems unnecessary to describe in detail the other experiments mentioned in Table I. The table, with its observations and footnotes, is self-explanatory. Although circulating virus was present in every source animal at the time of mosquito feeding, and was sometimes verified in the freshly fed mosquitoes, it was not always proved to be

TABLE II

Serial Transmission of Neurotropic Yellow Fever Virus in M. rhesus by Means of Stegomyia Mosquitoes

Passage of mouse virus in monkeys	Test monkey No.	Animal from which transfer was made	Manner of virus transfer	Mosquito lot used for transfer	Fever in test monkey	Serum infective for mice	Nervous manifestations	Death of test monkey	Encephalitis at death	Brain infective for mice	Subsequent immunity	Mosquito lots fed on test monkey	Day of experiment when fed	Infectivity of mosquitoes for mice: Injection immediately after original blood meal: Mouse groups	Route of injection	Infection proved	Later test of mosquitoes: Interval after original blood meal	Mouse groups	Route of injection	Infection proved
																	days			
I	A9	Mice (brains) Passage F167	Brain emulsion subcut.	*	−	+	−	−	−	*	+	661	1 (23 hrs.)	447	Subcut.	−	65	792	Ic.	−
														448	Ic.	−				
												663	2 (46 hrs.)	455	Ic.	+	64	792	Ic.	−
														456	Subcut.	−				
												665	5	477	Ic.	+	*	*	*	*
II	A10	A9	Serum ip.	*	+	− (8th day)	−	−	−	*	+	667	4 and 6	*	*	*	47	712	Bites	+
	B4	A9	Mosquito bites	663	−	*	−	−	−	*	−	688	4 and 6	*	*	*	*	*	*	*
	B6	A9	Mosquito bites	661	+	−	−	−	−	−	−	686	3 and 5	*	*	*	*	*	*	*
	C8	A9	Mosquito suspension subcut.	661 and 663	−	*	−	−	−	*	+?†	*	*	*	*	*	*	*	*	*

III	B8	A10	Mosquito bites	667	+	*	+	+‡	+	+		690	4 and 7	*	*	*	56	881	Ic.	–
IV	B11	B8	Blood ip.	*	–	*	–	–	–	*	+	*	*	*	*	*	*	*	*	*
	C1	B8	Brain emulsion ic.	*	+	+	+	+‡	+	+		693	3	*	*	*	53	888	Ic.	–
	C5	B8	Mosquito bites	690	+§	+	–	+‡	+	+		698	2	*	*	*	35	888	Ic.	–
V	C2	C1	Blood ip.	*	+	– (8th day)	–	–	–	*	+	694	4 and 8	*	*	*	59	897	Ic.	–
	C4	C1	Mosquito bites	693	+	+	–	–	–	*	+	699	5	*	*	*	42	898	Ic.	–
	C7	C5	Serum ip.	*	+	–	–	+‖	–	+		702	9	*	*	*	36	899	Ic.	–
	D2	C5	Mosquito bites	698	–	*	–	–	–	*	–	*	*	*	*	*	*	*	*	*
	D5	C1 C5	Mosquito suspension subcut.	693 698	+	– (11th day)	–	–	–	*	–	715	11	*	*	*	*	*	*	*
VI	C10	C2	Mosquito bites	694	+§	–	–	–	–	*	+	703 and 704	1, 2, 3, 4	*	*	*	53–54	918	Ic.	–
	C6	C4	Serum ip.	*	+	–	–	–	–	–	+	*	*	*	*	*	*	*	*	*
	D1	C4	Mosquito bites	699	+	*	–	–	–	*	–	*	*	*	*	*	*	*	*	*

– = negative. + = positive. Subcut. = subcutaneously. Ic. = intracerebrally. Ip. = intraperitoneally.

* No test made.

† Immunity questionable. Serum after 22 days protected mice; however, a test dose caused fever, but not death of monkey.

‡ Sacrificed when moribund.

§ Fever, perhaps caused by complicating tuberculosis.

‖ Animal collapsed and became moribund while being fed upon by mosquitoes.

TABLE II—*Concluded*

Passage of mouse virus in monkeys	Test monkey No.	Animal from which transfer was made	Manner of virus transfer	Mosquito lot used for transfer	Fever in test monkey	Serum infective for mice	Nervous manifestations	Death of test monkey	Encephalitis at death	Brain infective for mice	Subsequent immunity	Mosquito lots fed on test monkey	Day of experiment when fed	Infectivity of mosquitoes for mice						
														Injection immediately after original blood meal			Later test of mosquitoes			
														Mouse groups	Route of injection	Infection proved	Interval after original blood meal	Mouse groups	Route of injection	Infection proved
																	days			
VI	D4	C7	Mosquito bites	702	−	*	−	−	−	*	−	*	*	*	*	*	*	*	*	*
	D6	C2 C4 C7	Mosquito suspension subcut.	694 699 702	−	*	−	−	−	*	+	717	4, 8	*	*	*	*	*	*	*
VII	B6	C10	Mosquito bites	703–704	+	− (11th day)	−	−	−	*	−	*	*	*	*	*	*	*	*	*
	D7	D6	Blood ip.	*	+	*	−	−	−	*	−	718	2	*	*	*	*	*	*	*
	D8	D6	Mosquito bites	717	−	*	−	−	−	*	−	*	*	*	*	*	*	*	*	*

*No test made.

present in the mosquitoes at later dates. Many animals died from secondary infections following injection of mosquitoes, before yellow fever had time to develop.

The suspensions of mosquitoes used for intracerebral inoculation of mice in the experimental work listed in Tables I and II were dilutions of 1:20 to 1:50 in 10 per cent normal monkey (or normal human) serum, centrifuged but not filtered, unless otherwise stated under Observations.

In Table II is summarized an attempt to maintain mouse virus in *rhesus* monkeys, with intermediate passages through mosquitoes.

On Nov. 13, 1931, *M. rhesus* A9 was inoculated subcutaneously with 5 cc. of a 10 per cent suspension of infective brains, from the 167th passage of the French strain of yellow fever virus in mice. Serum from this monkey was injected intracerebrally into mice daily for 10 days following introduction of virus. That taken at 23 hours and at 46 hours proved to be infective; later injections of serum caused no deaths among the mice. Suspensions of freshly fed mosquitoes of the 2nd day and of the 5th day were infective for mice. The mosquitoes of the 2nd day later failed to transmit yellow fever; those of the 5th day were not tested further. Sera of the 1st and 2nd days (Nov. 14 and 15) were injected intraperitoneally into *M. rhesus* A10. The latter developed a high fever (maximum temperature 105.8°F.) on the 9th to 11th days after the injection; *M. rhesus* A9 never had a fever.

Mosquito Lot 667[1] was allowed to feed on *M. rhesus* A10 on Nov. 18 and 20, before the development of fever. The blood taken on Nov. 23 (1st day of fever) was non-infective for mice and gave a positive protection test against yellow fever virus. On Dec. 19 mosquito Lot 667 fed on *M. rhesus* B8. On the 4th day afterward fresh mosquitoes (Lot 690) engorged on the same monkey. On the 7th day (Dec. 26) the animal had a temperature of 105.4°F. Mosquito Lot 690 was allowed to feed once more. Blood transferred to *M. rhesus* B11 at this time caused no reaction, although the recipient was immunized by the injection. On Dec. 28 (3rd day of fever) *M. rhesus* B8 showed very definite neurological signs. Symptomatology included: paresis of arms, more marked on right; wrist drop; weakness; tremor of head and limbs, most marked in right arm; occasional nystagmus; incoordination, with difficulty in walking and in righting himself; crossing of arms when in sitting posture; hiding of head against side of cage or on floor; loss of fight; sharp, shrill cries. In the afternoon of the same day the temperature was found

[1] As a routine, female stegomyia mosquitoes are placed 180 to 200 to a cage. When 1 to 2 weeks old nearly all of them engorge when a monkey is introduced directly within their cage. It is safe to say that a batch or lot of freshly fed mosquitoes after the removal of the non-engorged and after due allowance for deaths since original counting and separation, consists invariably of well over 150 insects.

to be falling; tremor was accentuated; when placed on his feet the animal swayed, fell, and recovered with difficulty. In the certainty that death would occur during the night, the animal was killed with chloroform.

At autopsy *M. rhesus* B8 showed very few gross lesions. There was atrophy of subcutaneous fat, the spleen was slightly enlarged, and the liver was paler than normal; otherwise the organs were negative. Microscopically the liver showed infiltration of fat, particularly around the portal spaces; there appeared to be a slight parenchymatous degeneration, but no necrosis; no intranuclear inclusions were found. The kidney showed a slight cloudy swelling, with a few casts in the medulla. The spleen revealed occasional small necroses in the germinal centers of the follicles. Sections from the brain were of the greatest interest. There was no meningeal reaction. Inflammation was widely distributed throughout the brain, but the cerebellum was almost entirely spared. Cuffing of vessels was more marked in the brain stem. Tiny inflammatory foci and diffuse infiltration of leucocytes were noted in cerebral cortex, hippocampus, and pons. In some fields polymorphonuclear neutrophiles were prominent in addition to round cells. Degenerated and necrotic nerve cells were present. There was a relative or actual increase in glial cells. No intranuclear inclusions were found. The picture was that of a disseminated encephalomyelitis, fully as well developed as that produced by Lloyd and Penna (4), or as that produced by Sellards (5) and described by Goodpasture (6), in monkeys inoculated intracerebrally with neurotropic yellow fever virus.

Intracerebral inoculation of mice with brain substance from *M. rhesus* B8 killed two groups (twelve animals) in 4 to 6 days. Transfer of brain material from these to other groups brought down eleven out of twelve animals in 4 days. Thus the infection behaved like that produced by fixed neurotropic yellow fever virus. A protection test performed with this strain was unfortunately complicated by mouse typhoid. However, taking the six day reading as final, the result was as follows: Three groups (eighteen mice) given suspected virus emulsion and yellow fever immune serum showed one death on the 5th day, but no other animals sick up to the 6th day; two groups (nine mice surviving initial inoculation) given suspected virus and normal serum showed six dead and three sick; that is, all either dead or sick, on the 6th day.

Brain emulsion from *M. rhesus* B8 injected intracerebrally into *M. rhesus* C1 caused fever on the 3rd day. On the 7th day (Jan. 4, 1932) the animal had a falling temperature and fully developed clinical manifestations of encephalitis, in every way comparable to those produced by neurotropic yellow fever virus in the experiments of Lloyd and Penna at this laboratory. In the evening of the same day the monkey was obviously moribund and was killed with chloroform. Brain sections from this monkey showed a typical encephalomyelitis. The virus from the brain of *M. rhesus* C1 behaved in mice as the fixed neurotropic strain of yellow fever virus (mouse Groups R713 and R737).

On Jan. 16 mosquito Lot 690, which had obtained its infective blood meal on *M. rhesus* B8, fed on *M. rhesus* C5. 2 days later the monkey showed a tempera-

ture of 104°F. The blood at that time was infective for mice, killing all six of Group R823. On the 3rd day the temperature of *M. rhesus* C5 reached 105.8°F. There was a remission on the 6th day followed by fever until the 10th day, when the temperature again fell below 104°F. On the afternoon of that day the monkey was very weak, and evidently moribund; there were no definite neurological signs. Autopsy showed diffuse tuberculosis. Brain substance from this animal, both that filtered through Berkefeld N and that which was unfiltered, proved fully virulent to mice (Groups R829 and R830). The virus acted in all respects like the fixed neurotropic strain of yellow fever virus. Sections from the brain of *M. rhesus* C5 did not show as typical an encephalitis as those from Monkeys B8 and C1. There was a little hemorrhage into the pia-arachnoid over the medulla; a few vessels in the pons showed perivascular infiltration.

In the series an autopsy was performed upon but one other monkey, *M. rhesus* C7. This animal had received serum intraperitoneally from *M. rhesus* C5. Fever developed on the 9th day, at which time he was bound and put into a cage for mosquito feeding. While there he collapsed. He was removed in a moribund condition. The brain showed no perivascular infiltration, but occasional ganglion cells were degenerated (nuclear fading; cytolysis). Serum from *M. rhesus* C7 was non-infective for mice. However, brain emulsion filtered through Berkefeld candle V killed three out of six mice (Group R831) inoculated intracerebrally; subinoculated mice were killed promptly on the 4th day, precisely as with fixed neurotropic yellow fever virus.

In the four animals studied at autopsy neither the macroscopic nor the microscopic lesions were typical of those caused by viscerotropic yellow fever virus. In none of the animals did the liver show necrosis.

It was thought upon starting the experiment that repeated passage through mosquitoes might cause a reversion of the virus from the neurotropic to the viscerotropic type. Such a reversion was not demonstrated. Apparently the virus became progressively weaker and died out in the sixth passage from mice. It is quite possible that mosquitoes were not allowed to feed at the right time for picking up the maximum amount of virus. At the time of fever the blood of several monkeys was shown to be non-infective. Since it was impossible to rely upon fever as a guide, the time for feeding mosquitoes had to be chosen quite arbitrarily.

In Table I it will be noted that mosquitoes became infected by feeding on mice at a proper interval after the inoculation of the latter with large amounts of neurotropic virus. In Table II there is recorded the infection of mice by the bites of mosquito Lot 667, which obtained its infective blood meal from *M. rhesus* A10, 47 days previously. The

mouse group (R712) consisted of six baby mice about 2 weeks old. Three of the six became ill within the usual incubation period. From one showing complete paralysis of the hind legs, brain transfer was made to adult mice of Group R757. All of the latter were stricken on the 4th day, as is usual following intracerebral inoculation of fixed neurotropic virus.

Mosquito feeding on mice was accomplished with surprising ease. The animals were placed in cylinders about 1 inch in diameter, made of strong, wide meshed wire gauze. The ends of the cylinders were packed with cotton and strapped with adhesive plaster. Mosquitoes attacked the mice at once and engorged rapidly.

DISCUSSION

Lloyd and Penna (4) have already reported experiments demonstrating the fixed nature of the neurotropic French strain of yellow fever virus. The present experiments are confirmatory of this fundamental change in the nature of the virus. The adaptation to mouse brains does not signify an attenuation; under certain conditions the virus is still lethal for *rhesus* monkeys. However, the blood stream is only transiently invaded; no marked lesions are produced in liver and kidneys; and final localization occurs predominantly or entirely in the nervous system. This is true even after passage through mosquitoes. There may be means of inducing a reversion to the viscerotropic type, but such means have not yet been discovered by us.

SUMMARY

1. By the bites of stegomyia mosquitoes carrying neurotropic yellow fever virus, encephalitis has been produced both in white mice and in *rhesus* monkeys.

2. The fixed neurotropic strain of virus cannot be maintained in the mosquito host as well as can the viscerotropic strains. This is doubtless attributable in part to a smaller amount of virus ingested, because of paucity in the blood stream of the mammalian host.

3. These experiments furnish additional evidence that the long established neurotropic yellow fever virus has changed fundamentally from the parent French strain.

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