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FURTHER STUDIES ON THE EXPERIMENTAL TRANSMISSION OF YELLOW FEVER BY *SABETHES CHLOROPTERUS*

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In 1956 Galindo, Rodaniche and Trapido[8] reported the first experimental transmission of yellow fever by *Sabethes chlorpterus*. Virus was transmitted to one *Macaca mulatta* by the bites of 11 of these mosquitoes after an extrinsic incubation period of 27 to 34 days. A second effort to transmit to a rhesus monkey failed. Failure also was experienced in all attempts to recover virus by intracerebral injection of white Swiss mice, principally young adults, with suspensions of single mosquitoes or small pools. The need was felt for further investigation to clarify the potentialities of *S. chloropterus* as a vector of yellow fever. Since this initial report three additional experiments have been performed which have resolved many of the questions raised in earlier work. In general the same techniques were followed as were formerly employed.

MATERIALS AND METHODS

Virus strains. In the first of the three experiments here reported the Trinidad #4202 strain previously described was again employed. This virus was received in 1954 through the courtesy of Dr. Wilbur Downs, Director of the Trinidad Regional Virus Laboratory, as a suspension of the spleen of a howler monkey found dead in the forest. In the remaining two experiments, a yellow fever strain, C-10, isolated locally, was substituted. This virus was recovered from *Sabethes chloropterus* found naturally infected in Cerro Azul in September, 1956.

Mosquitoes. The *Sabethes chloropterus* were later generations of the same colonized strain used in the previous study. The wild stock for initiation of the colony was captured at Cerro La Victoria in Panama in 1955 and was maintained in a closed insectary, the temperature of which varied between 23°C and 30°C and the relative humidity between 70 and 100%. Infected mosquitoes were kept in large cages in a separate room, completely isolated from the breeding stock and the outside environment by double screen doors.

Serologically negative, healthy, young, rhesus monkeys were employed as the source of infective blood for the initial mosquito feedings. These monkeys were injected subcutaneously with yellow fever virus 2 to 4 days prior to exposure to *S. chloropterus*. The virus content of the blood was titrated in adult mice daily. The monkeys were immobilized on a special board which was placed inside the mosquito cage. Miltown was administered in the first two experiments to tranquilize the animals but its use was discontinued due to fear that it might be having a deleterious effect on the mosquitoes, which showed an unusually high mortality. Great difficulty was experienced in early experimental work in inducing *Sabethes* to feed on monkeys. It was subsequently found[1] that the peak of biting activity does not occur until the second or third week, or even as much as 30 days, after emergence. The initial difficulty was overcome by using older females.

After suitable extrinsic incubation periods, the *Sabethes* were refed on normal monkeys. Young *Macaca mulatta* were used for this purpose in the first two experiments. In the third, juvenile black spider monkeys, *Ateles geoffroyi grisescens* Gray, captured in the wild stage and giving negative protection tests, were substituted.

Mosquitoes found dying or recently dead after an opportunity to become infected were ground with rhesus-serum-saline solution, either singly or in small pools, and injected intracerebrally into white Swiss mice. The quantity of diluent was always 0.6 ml regardless of whether single or pooled insects were used, as this was considered a minimal quantity for our purpose.

Mice becoming ill after an incubation period of more than 4 days were sacrificed. Smears stained with Giemsa, and bacteriological cultures of cerebral tissue, were made and the brain was then emulsified for injection into a group of five normal mice and five mice previously im-

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munized against Theiler's French neuroadapted strain of yellow fever virus. Identification of virus was considered adequate when smears showed no microorganisms, cultures were negative for bacteria, and the immune animals were still alive and well after the nonimmunes had sickened or died. Mice were kept under observations for a period of 28 days. At the end of this time survivors were reinjected intracerebrally with 10,000 LD/50 of French neuroadapted virus to discover possible inapparent infections.

RESULTS

Experiment I

In the first of the transmission experiments here reported (see Table 1) *S. chloropterus* were allowed to feed on a rhesus monkey on the 2nd, 3rd and 4th days after inoculation with the Trinidad #4202 strain in its second monkey passage, when the blood titers of virus were $10^{-5.2}$, $10^{-5.8}$ and $10^{-4.3}$, respectively. The mosquitoes fed very poorly, as this was previous to the use of older females. After an extrinsic incubation period of 32 to 34 days, the infected mosquitoes were again exposed to a normal rhesus but only three fed. The result was completely negative. The animal did not develop fever, did not circulate virus and failed to show immunity after a 30-day observation period. Only 13 *Sabethes* were available for mouse inoculation. Virus was not recovered in infant mice from any of them.

Experiment II

In the second transmission experiment (see Table 1) the local C-10 strain of yellow fever virus in its second monkey passage was used. Two lots of *S. chloropterus* were allowed to feed on a normal rhesus monkey, one on the 3rd day after subcutaneous injection, when the titer of circulating virus was $10^{-7.2}$, and the other on the 4th day when the titer was $10^{-5.3}$. The mosquitoes fed well. Specimens found dying or recently dead during the subsequent 27 days were used for mouse injection. (See mosquito lots 1 and 2 in Table 2.) Then all survivors of both lots were combined and allowed to feed on a normal young *Macaca mulatta*. This animal was exposed to the bites of the infected mosquitoes during a 3-day period after an extrinsic incubation period of 27 to 30 days. A total of 25 fed. As the monkey remained afebrile and apparently healthy during the succeeding week, it was re-exposed to the bites of the same mosquitoes which had previously engorged, the extrinsic incubation period at this time being 34 to 37 days. Fourteen mosquitoes refed. The rhesus began to circulate virus 5 days later and continued to show viremia for 3 days. It was febrile 6 days after the last exposure

TABLE 1

Results of attempted transmission of yellow fever virus to monkeys by bite

Virus strain	Titers of circulating virus in infective meals	Extrinsic incubation period (*days*)	Monkey used for bite transmission	Number of mosquitoes refeeding	Results
Experiment I					
Trinidad #4202	$10^{-5.2}$, $10^{-5.8}$ and $10^{-4.3}$*	32–34	Rhesus #1	3	Negative
Experiment II					
C-10	$10^{-5.3}$ and $10^{-7.2}$†	28–29	Rhesus #2	25	Positive‡
		35–36		14	
C-10	$10^{-5.3}$ and $10^{-7.2}$	42–43	Rhesus #3	13	Positive
Experiment III					
C-10	$10^{-6.4}$	29	Black spider #1	10	Negative
C-10	$10^{-6.4}$ and $10^{-6.5}$†	37–38	Black spider #2	14	Negative
C-10	$10^{-6.4}$	42	Black spider #3	8	Negative
C-10	$10^{-6.4}$ and $10^{-6.5}$	53–54	Black spider #4	8	Negative

* Pool of mosquitoes fed on 2nd to 4th days after inoculation of source monkey.

† Pool of mosquitoes fed on 3rd and 4th days after inoculation of source monkey.

‡ Time of infection uncertain as animal was exposed more than once.

TABLE 2

*Results of inoculation of infant mice with suspensions of individual mosquitoes or small pools**

Mosquito lot	Titer of virus in infective meal	Total numbers and percentages positive of mosquitoes according to weeks after feeding*									
		1–2 weeks		3–4 weeks		5–6 weeks		7–9 weeks		Totals	
		Tot.	% pos.	Tot.	% pos.	Tot.	% pos.	Tot.	% pos.	Tot.	% pos.
Experiment II											
Lot 1	$10^{-7.2}$	31	(16–26)†	5	(40–60)	—	—	—	—	36	(20–30)
Lot 2	$10^{-5.3}$	65	(1–6)	20	(5–25)	—	—	—	—	85	(3–14)
Lots 1 & 2	$10^{-5.3}$ and $10^{-7.2}$	—	—	—	—	19	(16–80)	12	(58)	31	(32–74)
Experiment III											
Lot 1	$10^{-6.4}$ and $10^{-6.5}$	32	(34)	18	(44)	8	(62)	19	(68)	77	(48)
Totals		128	(13–18)	43	(26–37)	27	(30–80)	31	(64)	229	(25–37)

* Data from experiment I are not included as all results were negative.

† Pools were included. Percentage positive depends on whether one or all mosquitoes in the pool are considered positive.

and showed irregular temperature rises, between 102.6° and 104.6° F., for a period of 5 days. It did not die, however. The blood possessed a high titer of protective antibodies 28 days after the first exposure, 19 days after the last. Negative reactions had previously been obtained with blood drawn prior to exposure and on the 14th day after the first exposure. Unfortunately, it cannot be determined definitely when the monkey acquired its infection, but it seems highly probable that this occurred during the second exposure and that the prolongation of the extrinsic incubation period was the crucial factor.

A second *Macaca mulatta* was then exposed to the bites of these same *S. chloropterus* for 1 day, after an extrinsic incubation period of 43 to 44 days. Thirteen engorged. This animal did not develop fever but became prostrate and died 7 days later when it was being prepared for a second exposure. Virus was recovered from its blood at this time. Thus, transmission was successfully effected by *S. chloropterus* to both rhesus monkeys under experimental conditions.

In the course of this experiment, 152 mosquitoes were used for mouse inoculation. Two- to 3-day-old mice were employed exclusively, 0.02 ml of the inoculum being delivered intracerebrally. Mosquitoes were triturated for injection either singly or in small pools of less than five. The use of some pools prevented, of course, the exact assessment of positive results. Examination of Table 2 will show that a positive virus isolation in infant mice depends on two factors: (1) the length of the extrinsic incubation period and (2) the titer of virus in the infective meal. After an extrinsic incubation period of 1 to 4 weeks, 20 to 30% of the mosquitoes (depending on whether only one or all of the mosquitoes in the positive pools are counted as positive) which had engorged on blood with a titer of $10^{-7.2}$, contained sufficient virus to pass the threshold of infection in infant mice; whereas, only 3 to 14% of those fed on blood with a titer of $10^{-5.3}$ demonstrated this capacity. A definite rise in the proportion of mosquitoes containing sufficient virus to infect infant mice may be observed in all groups as the extrinsic incubation period becomes more prolonged.

Experiment III

In the third experiment *S. chloropterus* were fed on a *Macaca mulatta* on the third and fourth days after subcutaneous injection with the C-10 strain of yellow fever virus, when the blood titers were $10^{-6.4}$ and $10^{-6.5}$, respectively. (See Table 1.) Juvenile black spider monkeys, captured in the wild state and housed at the Laboratory, were used instead of rhesus for bite-transmission attempts. Four of these *Ateles* were exposed to the attacks of the mosquitoes after incubation periods of 29, 37 to 38, 42 and 53 to 54 days, respectively.

Between eight and 14 mosquitoes engorged on each animal. However, no transmission was effected. None of the spider monkeys showed fever or any other symptom, circulated virus or developed protective antibodies. Other data accumulated in previous work indicate that Panama's black spider monkey has a high natural resistance against this disease.

A total of 77 mosquitoes was used for intracerebral injection of infant mice. All mosquitoes were ground and injected individually in order to obtain exact figures on the number of positives. A total of 37 (48%) contained sufficient virus to pass the threshold of infectivity of infant mice. (See Table 2.) The virus titers of the two infective meals were so closely similar that no attempt to classify the insects according to this criterion has been made. Again we may observe a steady increase in the proportion of positives with lengthening of the extrinsic incubation period; *i.e.*, 34% of 32 after 1 to 2 weeks of incubation and 68% of 19 after 7 to 9 weeks. Minimal quantities of virus were present, however; seldom sufficient to produce infection in all the mice of the inoculated groups. Four of the more strongly positive specimens were given a further 10-fold dilution after storage in CO_2 ice, and injected into infant mice, but none was able to infect all mice in this higher dilution and one failed to infect any. It is thus obvious that, even under the most favorable of circumstances, *Sabethes chloropterus* harbors only traces of virus.

DISCUSSION

An experiment of this kind always includes a number of uncontrollable variables which render accurate evaluation difficult. Certain factors entering into the complex have been clarified, however. It is now certain that *Sabethes chloropterus* may harbor small quantities of virus for life and transmit by bite. It is, nevertheless, by no means as efficient a vector as *Haemagogus*. We have obtained bite transmission only when the animal was attacked by a relatively large number of mosquitoes, whereas with *Haemagogus mesodentatus mesodentatus* transmission was readily obtained by the bite of a single specimen. There is also considerable individual variation in the capacity to transmit among the members of even a long-colonized population of *Sabethes*. Further study is required to determine what influence, if any, adaptation to colony life may exert.

In order to obtain transmission by this species there are certain requirements, namely:

1) an appropriate virus strain; 2) a high titer of virus in the infective meal; 3) a prolonged extrinsic incubation period; 4) a highly susceptible host.

The vector potentialities of *Sabethes chloropterus* have particular importance because this mosquito is long-lived and survives in fair numbers over the dry season. If it could harbor virus during this period and later transmit by bite, it would provide a solution to the perplexing problem of the survival of virus after the rains stop. Galindo and Trapido have been the principal advocates of this theory which was advanced in publications made in 1950, 1951 and 1956,[2, 3, 4] on epidemiological grounds. Other supporting evidence for the possible importance of *S. chloropterus* as a vector of yellow fever consists in the fact that this species has been found infected in nature. Shannon, Whitman and Franca[7] isolated yellow fever from a mixed lot of *Sabethoides*, *Limatus*, *Wyeomyia*, and *Trichoprosopon* (in which *S. chloropterus* was included), but it is not known which of the species was infected. More recently Rodaniche and Galindo[5] and Rodaniche, Galindo and Johnson[6] have reported the isolation of yellow fever virus from pure pools of *Sabethes chloropterus* captured in Guatemala and Panama during periods of epizootic activity in those countries.

In 1957 an attempt was made to recover virus from this species over the dry season. Virus had been active at Cerro Azul in Panama, near the end of the rainy season, in August, September and October, 1956. It was recovered from mosquitoes seven times during this period, including twice from *Sabethes chloropterus*. Captures were continued during the dry months, a total of 243 specimens being obtained (79 in January, 48 in February, 38 in March, 46 in April and 32 in May). However, no yellow fever virus was recovered.

SUMMARY

Confirmation is provided of previously reported experimental transmission of yellow fever virus by the bites of *Sabethes chloropterus*. This mosquito is a relatively inefficient vector, however, and requires a suitable virus strain, a high titer of virus in the infective meal, a prolonged extrinsic incubation period and a highly susceptible host, to be successful.

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