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COMMUNICATIONS

ZIKA VIRUS

(I). ISOLATIONS AND SEROLOGICAL SPECIFICITY

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The isolation of filterable viruses from mosquitoes taken in Uganda has already been recorded on several occasions. Two of the agents so recovered, although well known, had not previously been identified by isolation from mosquitoes in Uganda, viz. yellow fever virus (MAHAFFY et al., 1942 ; SMITHBURN and HADDOW, 1946 ; SMITHBURN et al., 1949) and Rift Valley fever virus (SMITHBURN et al., 1948). A third which was called Mengo encephalomyelitis (DICK et al., 1948) (now known to be identical with Columbia SK,MM and encephalomyocarditis viruses (DICK, 1949 ; WARREN et al., 1949), has been isolated on several occasions from *Taeniorhynchus* spp. (DICK et al., loc. cit., DICK and HADDOW, (unpublished)). GILLETT and DICK (unpublished) have, however, failed to transmit this agent in the laboratory by three species of *Taeniorhynchus*. The isolation of three hitherto unknown, filterable viruses secured from wild mosquitoes in Uganda has been described, viz. Bunyamwera virus (SMITHBURN et al., 1946), Semliki Forest virus (SMITHBURN and HADDOW, 1944), Ntaya virus (SMITHBURN and HADDOW, 1951) ; the description of a fourth, Uganda S virus, is to be published (DICK and HADDOW). The purpose of this communication is to describe the isolation of yet another virus which is believed to be hitherto unrecorded. Particular interest attaches to the recovery of this virus, partly because it was encountered on two occasions separated by

9 months, and partly because it was isolated independently by G.W.A.D. and later by S.F.K. employing different methods, both of which were being used primarily in the search for yellow fever virus in nature. This virus has been called Zika virus after the geographical name of the area from where the isolations were made.

MATERIALS AND METHODS

The mice used in all experiments were descended from the stock of Swiss mice from Carworth Farms, New York. A group of mice consisted of six, and unless otherwise stated all mice used in the experiments recorded in this paper were 35 to 42 days old. The quantity of the intracerebral inoculum for all mice was 0.03 ml.

ISOLATION OF VIRUS

Locality.

About 7 miles north east of this Institute there is a forested area called Zika. This area of forest consists of a narrow, dense belt of high but broken canopy growth with clumps of large trees. It lies along the edge of a long arm of Lake Victoria from which it is separated by a papyrus swamp. The greater part of the forest runs parallel with the Entebbe-Kampala road; there is a narrow stretch of grassland between the forest and the road. The forest at no place is more than 500 yards wide. It is about 1 mile in length and is almost continuous with scattered forest which in turn joins the forest at Bujuko on the Kampala-Fort Portal road. A detailed description of this forest has recently been prepared by BUXTON (in press).

Owing to the relatively high incidence of immunity to yellow fever among the monkeys on the Entebbe peninsula, Zika was chosen as one of the yellow fever study areas. As an implementation of the sentinel rhesus monkey programme commenced in Bwamba in 1946 (HADDOW et al., 1948), rhesus monkeys were located in cages on wooden platforms in the canopy of trees throughout the length of the Zika Forest.

First Isolation.

In April 1947, six sentinel platforms were in use at Zika. The temperatures of the rhesus monkeys on the platforms were taken daily. On 18th April, 1947, it was reported that the temperature of one of these monkeys — Rhesus 766 — was 39.7°C. (Fig. 1). On 19th April its temperature was recorded as 40°C. On the following day it was brought to the laboratory at Entebbe. It was kept under observation for 30 days but showed no abnormality other than the slight pyrexia recorded in Fig. 1.

A sample of blood was taken from Rhesus 766 on 20th April, the 3rd day of fever. The serum was injected intracerebrally (0.03 ml.) and intraperitoneally (0.06 ml.) into groups of mice and into another monkey, Rhesus 771 (0.75 ml. subcutaneously). The mice which were inoculated intraperitoneally with the serum showed no abnormality during an observation period of 30 days. All of those inoculated intracerebrally showed signs of sickness on the 10th day after inoculation. A filterable transmissible agent was isolated from the brains of these sick mice. The further adaptation to mice of this agent isolated from the serum of Rhesus 766 is described in the next communication (DICK, 1952).

The rhesus monkey (771) which was inoculated with serum of Rhesus 766 showed no elevation of temperature above its normal range, and no other abnormalities during an observation period of 23 days.

As will be described later, the agent isolated from the serum of Rhesus 766 which will be referred to as Zika virus (766 strain) was neutralized (a) by convalescent serum taken from Rhesus 766, 1 month (20th May) after the febrile episode (Fig. 1), and (b) by serum taken from Rhesus 771, 35 days after inoculation with

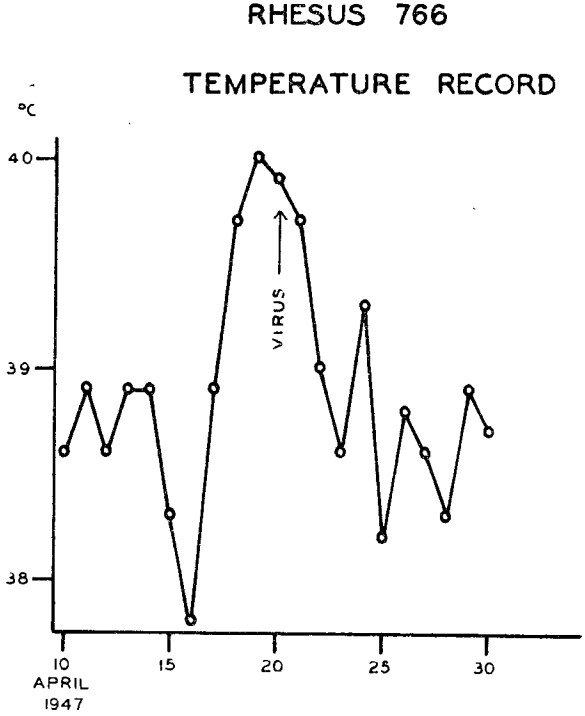

FIG. 1

the serum of Rhesus 766, but not by (c) pre-infection sera from these monkeys. (Table I). There was thus conclusive evidence that the agent isolated in mice had come from the serum of the sentinel Rhesus monkey (766).

Second Isolation.

In January 1948, in an attempt to isolate yellow fever virus from mosquitoes, a series of 24-hour catches was made in Zika Forest. The tree-platforms referred to above were employed in making these mosquito catches using methods which have already been described (HADDOW et al., 1947). The mosquitoes taken each

day were brought to the laboratory in Entebbe. After identification, the following grouping according to species or genera was made :

1. *Aedes* (*Stegomyia*) *africanus* Theobald.
2. Other arboreal *Aedes* spp.
3. Non-arboreal *Aedes* spp.
4. *Taeniorhynchus* spp.
5. *Eretmapodites* spp.
6. Other mosquitoes.

Because of its suspected implication in the forest cycle of yellow fever (HADDOW et al., 1948), *A. africanus* was treated individually as a species. After the grouping of the mosquitoes had been effected, the mosquitoes were rendered inactive by placing the tubes in which they were contained in a refrigerator at approximately 4°C.

From 5th to 20th January, nine lots of *A. africanus* were secured, and each was ground up in 10 per cent. serum-saline. The quantity of the serum-saline diluent employed was the minimum quantity which would make a satisfactory suspension of each lot of mosquitoes. These suspensions were centrifuged at approximately 2,000 r.p.m. for 15 minutes. An aliquot of each suspension was passed through a Seitz E.K. pad, and groups of mice were inoculated intracerebrally with the unfiltered supernates and with the Seitz filtrates.

This account is particularly concerned with the mosquito catch of 11th to 12th January, 1948. Eighty-six *A. africanus* (lot E/1/48) were taken, and were treated as described above. All of the six mice which were inoculated with the unfiltered supernate of this lot of mosquitoes appeared inactive on the 7th day after inoculation. Of those inoculated with the Seitz filtrate one mouse died on the 6th day and one appeared sick on the 14th day. Successful transfers were made with brain suspensions from mice inoculated with the unfiltered and with the Seitz-filtered suspensions of *A. africanus*. Evidence will be presented which demonstrates that the virus isolated from the suspensions of the E/1/48 lot of *A. africanus* was a strain (E/1 strain) of Zika virus. The history of the adaptation of this strain of virus is described later (DICK, 1952).

After the groups of mice had been inoculated with the suspensions of each lot of *A. africanus* taken in the January, 1948, mosquito-catches, the remaining portions of the unfiltered supernates and Seitz filtrates of all lots of *A. africanus* were inoculated subcutaneously into Rhesus 758. Between 5th and 20th January this monkey received nine inoculations of the remainder of suspensions of *A. africanus* totalling 607 specimens. The number of mosquitoes in each lot and the days of inoculation are indicated in Fig. 2. The total quantities of the unfiltered and Seitz-filtered suspensions inoculated into Rhesus 758 were 3.22 ml. and 0.61 ml. respectively. The quantities of the suspensions of the E/1/48 lot of mosquitoes available for inoculation on 13th January were 0.4 ml. of the unfiltered supernate and 0.1 ml. of the Seitz filtrate.

Rhesus 758 showed no abnormality as a result of the inoculation of the suspensions of *A. africanus*. The slight elevations of temperature recorded on 24th January and 5th February (Fig. 2) are not significant. Because of the appearance of sickness on the 7th day (20th January) in mice inoculated with the unfiltered supernate of the E/1/48 lot of *A. africanus*, a sample of blood was withdrawn from Rhesus 758 on the 21st, 22nd and 23rd January, i.e. on the 8th, 9th and 10th days after inoculation with the E/1/48 lot of *A. africanus*. The sera from these samples of blood were inoculated intracerebrally into groups of mice. Of the six mice inoculated with serum taken on 21st January, one was sick

on the 10th day and two were found dead on the 19th and 20th days. Successful transfers were made with a Seitz-filtered suspension of the brain of the sick mouse. Of the six mice inoculated intracerebrally with serum taken from Rhesus 758 on the 22nd January, one had questionable signs of sickness on the 12th day after inoculation and was found dead on the 13th day ; another mouse was paralysed on the 20th day. Successful transfers were made with a Seitz-filtered suspension of the brain of the dead mouse. (The paralysed mouse proved to be an example of Theiler's encephalomyelitis.) All the mice inoculated with serum taken from Rhesus 758 on 23rd January remained well.

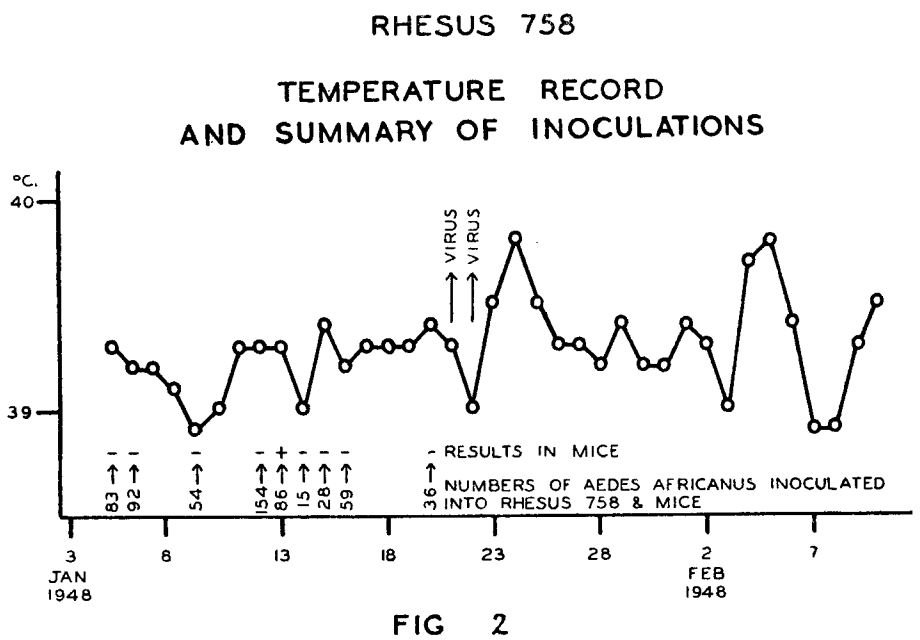

FIG 2

It was subsequently shown by neutralization tests that Rhesus 758 had developed neutralizing antibody to (a) the agent isolated from its serum, (b) the E/1 strain of virus isolated directly in mice from *A. africanus* and (c) to the 766 strain of Zika virus. These tests, which will be described in detail, indicated that a strain of Zika virus (Zika virus 758 strain) was isolated from the serum of Rhesus 758 which had been inoculated with suspensions of *A. africanus* (Fig. 2).

SEROLOGICAL STUDIES

Neutralization Tests.

Preliminary investigations, which will not be discussed here in detail, showed that adult mice were relatively insusceptible to intraperitoneal inoculations of

Zika virus. An " intracerebral " neutralization test based on the method described by THEILER (1933) was accordingly used for neutralization tests with Zika virus.

The technique employed in all neutralization tests, except in the case of a few tests made in 1948, was as follows : ten-fold dilutions of the lightly centrifuged supernate of a 10 per cent. suspension of infected mouse brains were made in serum-saline. To 0.3 ml. quantities of the sera to be tested were added 0.3 ml. of one of the dilutions of mouse brain virus as indicated in Tables I to IV. The serum virus mixtures were incubated at 37.5°C. for 2 hours prior to the intracerebral inoculation of groups of mice with each mixture.

In the other neutralization test technique, uncentrifuged virus suspensions of infected mouse brains were diluted to 10^{-2} and 0.15 ml. of this was mixed with 0.3 ml. of the sera to be tested. The further procedures of incubation and inoculation were as described above.

Demonstration of antibody in sera of infected or inoculated rhesus monkeys.

The development of antibody in the convalescent serum of Rhesus 766 and in the serum of Rhesus 771 was demonstrated by the neutralization test documented in Table I. In that test the first neutralization technique described was employed using infected brains of the ninth passage of the 766 strain of virus. Because of the low mortality and in some cases recovery of mice inoculated with early-passage virus, morbidity rather than mortality ratios were used in estimating the virus titres. It will be seen that neutralization indices of over 8,000 and over

TABLE I. Intracerebral neutralization tests with sera of Rhesus 766 and 771.

Virus	Serum			Morbidity ratios of mice inoculated with serum + virus dilution 10^{-1} 10^{-2} 10^{-3} 10^{-4}	MD_{50}*†	Log units neutralized
	Rhesus No.	Date bled	Descriptions			
9th passage	766	18.II.47	Prior to going to Zika as sentinel	6/6 6/6 6/6 6/6	4.5 or >	—
	766	20.V.47	31 days after onset of pyrexia	1/6 0/6 0/6 0/6	0.6 or <	3.9 or >
	771	8.IV.47	Pre-inoculation with Rhesus 766 serum	6/6 6/6 6/6 6/6	4.5 or >	—
	771	25.VI.47	36 days after inoculation with Rhesus 766 serum	0/6 0/6 0/6 0/6	0.5 or <	4.0 or >

* (1) 50 per cent. morbidity end point.
† (2) All end points are calculated by the method of Reed and Muench (1938).

10,000 were obtained with convalescent serum from Rhesus 766 and 771 respectively.

Using the second neutralization test technique described, it was shown that a 10^{-2} suspension of the eighth passage of the strain of virus isolated from the serum of Rhesus 758 (taken 21st January, 1948) was completely neutralized (a) by

serum taken from Rhesus 758 32 days after inoculation with the suspension of the E/1/48 lot of *A. africanus*, (b) by late convalescent serum from Rhesus 766, but not by (c) serum taken from Rhesus 758 on 3rd January, 1948, prior to the inoculations of the suspensions of *A. africanus* (Fig. 2). A similar experiment using the seventh passage of the E/1 strain of virus showed that a 10^{-2} suspension of infected mouse brain was neutralized by convalescent serum from Rhesus 758. From these tests it was concluded that Rhesus 758 had developed antibody both to the strain of virus isolated from its serum and to the strain isolated directly in mice from the E/1/48 lot of *A. africanus*.

Identity of sentinel monkey and A. africanus *strains.*

The identity of the 766 and E/1 strains of Zika virus was established by the neutralization tests outlined in Table II. In these tests and in all subsequent neutralization tests described in this communication, the first technique described was employed.

It will be seen from Table II that there was reciprocal neutralization of the E/1 strain and 766 strain of virus by the late convalescent serum of both Rhesus 758 and Rhesus 766.

Serological specificity.

Cross neutralization tests were made with Zika virus, with yellow fever and Hawaii dengue viruses and with the FA and GD VII strains of Theiler's encephalomyelitis virus. In the case of other viruses, known antisera were tested against dilutions of Zika virus only.

Yellow Fever. Soon after the isolation of the first strain of Zika virus from Rhesus 766, it was shown that samples of serum taken from Rhesus 766 (a) before it was sent to Zika, and (b) during convalescence, had no neutralizing effect on the French neurotropic strain of yellow fever virus when these sera were tested in the yellow fever neutralization test as modified by SMITHBURN (1945). Furthermore, yellow fever hyperimmune serum failed to neutralize early passage Zika virus (766 strain) in tests employing the intracerebral test technique already described. Similarly, pre-inoculation and convalescent sera from Rhesus 771 failed to show any neutralization of yellow fever virus and the E/1 strain of virus was not neutralized by yellow fever hyperimmune serum. There was thus good evidence that Zika virus was not related to yellow fever.

Dengue Virus. The results of cross neutralization tests with Zika and Hawaii dengue viruses are presented in Table III. In the test using Zika virus, the intracerebral technique already described was employed. All sera in this and subsequent neutralization tests to be described, were inactivated at 56°C. for 30 minutes before being tested. The dengue immune serum (M 4897) was supplied by Dr. K. C. SMITHBURN (Division of Medicine and Public Health

TABLE II. Cross neutralization tests with the E/1 and 766 strains of Zika virus.

Virus Strain	Serum Rhesus	Serum Date	Serum Description	Mortality or morbidity of mice* inoculated with serum + virus dilution 10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	LD_{50} or MD_{50}*†	Log units neutralized
E/1 99th passage	651	5.VI.50	Normal	—	—	6/6	6/6	5/6	3/6	0/6	6.8	
	758	2.VI.50	E/1 strain immune	—	—	4/6	1/6	0/6	0/6	—	4.4 or >	2.4 or <
	766	10.V.50	766 strain immune	—	—	0/6	0/6	0/6	0/6	—	3.5 or <	3.3 or >
766 24th passage	984	10.V.51	Normal	—	6/6	4/6	0/6				4.2	
	758	10.V.51	E/1 strain immune	1/6	0/6	0/6	—				1.6 or <	2.6 or >
	766	10.V.51	766 strain immune	0/6	0/6	0/5	—				1.5 or <	2.7 or >

* The titres are calculated on mortality ratios in the case of the test using the E/1 strain and on morbidity ratios in the test using the 766 strain.

† See footnote (2) Table I.

TABLE III. Cross neutralization tests with Zika and Hawaii dengue viruses.

Virus	Serum Description	Serum Rhesus No.	Survival ratios of mice inoculated with serum plus virus dilution 10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	LD_{50}†	Log Units neutralized
Zika (E/1) 108th passage	Normal	970		0/6	0/5	2/6	5/6	5/6	6.6	—
	Dengue immune	M. 4897		0/6	0/6	1/6	5/6	—	7.0 or >	—
	Zika immune	766		6/6	6/6	6/6	—	—	3.5 or <	3.1 or >
Hawaii dengue 82nd passage	Zika non-immune	728	0/8	0/8	0/8	5/8	7/8		5.9 or >	
	Zika immune	766	0/8	0/8	0/8	5/8	8/8		5.8	0.1 or >
	Dengue immune	808	7/8	8/8	8/8				2.6 or <	3.3 or >

† See footnote (2) Table I.

Laboratories of The Rockefeller Foundation). It was demonstrated in this laboratory that this serum neutralized 3.2 log units of 83rd-passage mouse brain Hawaii dengue virus when tested intracerebrally in 7-day-old mice by the method described.

It will be seen in Table III that the Hawaii dengue immune serum had no neutralizing effect on 108th mouse brain passage Zika virus.

The strain of Hawaii dengue virus employed (Table III) was supplied as a lyophilized 5 per cent. mouse brain suspension of 77th mouse passage virus by Dr. M. THEILER (Division of Medicine and Public Health Laboratories of The Rockefeller Foundation). After five intracerebral passages in 7-day-old mice in this laboratory, two brains of the 82nd mouse passage virus were used for a neutralization test. The brains were emulsified in the whole serum of a dengue non-immune monkey to make a 10 per cent. suspension. Serial 10-fold dilutions of this suspension were made with that serum as indicated in Table III. The dengue anti-serum used in this test was supplied by Dr. A. B. SABIN (the Children's Hospital, Cincinnati, Ohio) as lyophilized rhesus monkey serum. This serum was rehydrated and inactivated immediately before use. The subsequent procedures were the same as in the neutralization tests already described except that (a) the serum-virus mixtures were incubated for only 1 hour at 37.5°C., and (b) the inoculations were made intracerebrally into 7-day-old mice. It may be seen in Table III that Zika immune serum had no significant neutralizing effect on 82nd-passage Hawaii dengue virus.

Theiler's encephalomyelitis virus. The FA and TO strains of Theiler's mouse encephalomyelitis virus were received from Dr. M. THEILER and the GD VII strain from Dr. H. A. HOWE. Antiserum to the FA strain was prepared by repeated inoculations of a rabbit (375) with mouse brain virus and antiserum to the GD VII strain was prepared in cotton-rats by a similar method. It was shown that while antisera to the FA and GD VII strains neutralized 1.4 and 1.6 log units of these viruses respectively, they gave no significant neutralization of Zika virus. Reciprocally it was demonstrated that there was no significant neutralization of the FA, GD VII or TO strains of virus by antiserum to Zika virus. In general, neutralization tests with Theiler's virus are unsatisfactory. Apart from the immunological evidence, the pathogenicity of Zika virus indicates that it is not identical with strains of Theiler's encephalomyelitis of mice.

Test with other antisera. Neutralization tests were made with the antisera to the viruses listed in Table IV and to Mengo encephalomyelitis and Uganda S viruses. Antisera to Bunyamwera, Semliki Forest, Bwamba fever, Ntaya, Mengo encephalomyelitis, Ilhéus and Uganda S viruses were prepared at this institute by the inoculation of animals with mouse brain suspensions of these agents. Antisera to Eastern equine, Western equine, St. Louis and Japanese B encephalitis viruses were prepared in New York by Dr. K. C. SMITHBURN (Division of Medicine and Public Health Laboratories, The Rockefeller Foundation). All of these sera

TABLE IV. Neutralization tests with antisera of some neurotropic viruses.

Serum	Source of serum	Survival ratios of mice inoculated with serum plus virus dilution						LD_{50}†	Log units neutralized
		10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}		
Normal monkey	Rh.*651		0/6	3/5	3/4	5/6	6/6	5.2	—
Normal human	A.J.H.		0/6	3/6	4/6	6/6	6/6	5.3	—
Immune to virus :									
Bunyamwera	Rh.415		0/6	3/6	4/5	6/6	5/5	5.1	0.1
Semliki Forest	*Aethiops* 255		0/6	1/6	4/5	6/6	6/6	5.5	—
Bwamba fever	668		0/6	1/6	1/6	6/6	6/6	6.3	—
Ntaya	Rh.282		0/6	1/6	5/6	6/6	5/5	5.5	—
West Nile	Rh.4269		0/6	3/6	2/6	5/6	6/6	6.0	—
Ilheus	Rh.855		0/5	1/5	5/5	5/5	5/5	5.3	—
Eastern equine e.‡	Rh.M.4904		0/6	0/6	4/6	6/6	5/5	5.7	—
Western equine e.	Rh.M.4903		0/6	1/6	5/6	6/6	5/6	5.5	—
Venezuelan equine e.	Guineapig 2		0/6	1/6	5/6	6/6	6/6	5.5	—
St. Louis e.	Rh.M.4896		1/6	1/6	6/6	6/6	6/6	5.3 or <	—
Japanese B. e.	Rh.M.4913		0/6	1/6	5/6	6/6	6/6	5.5	—
Lymphocytic choriomeningitis	Guineapig		0/6	2/6	4/6	4/4	6/6	5.5	—
B virus (Canfield)	Rh.1203		0/6	4/6	4/6	6/6	6/6	5.0	0.2
Louping ill	Horse		0/6	3/6	5/5	6/6	5/6	5.0 or >	0.2 or <
Zika	Rh.766	6/6	6/6	6/6	6/6	6/6	—	2.5 or <	2.7 or >

† See footnote Table I.
* Rh. — rhesus.
‡ e. — encephalitis.

neutralized more than 2 logs of the homologous virus. Lymphocytic choriomeningitis antisera were provided by Dr. F. O. MacCallum (Virus Reference Laboratory, Colindale, London) and by Dr. T. P. Hughes (former staff member, The Rockefeller Foundation), Venezuelan equine encephalitis antiserum by Dr. P. K. Olitsky (The Rockefeller Institute for Medical Research, New York), B. virus (Canfield) antiserum by Dr. A. B. Sabin (The Children's Hospital, Cincinnati, Ohio) and louping ill antiserum by Dr. D. G. ff. Edward (The Wellcome Veterinary Research Station, Frant, England). Acknowledgement is made to all who kindly supplied these specific neutralizing antisera.

All the antisera listed in Table IV were tested against dilutions of the 99th passage of the E/1 strain of Zika virus. The mice employed in that experiment, which is recorded in Table IV, were 42 to 45 days old.

Neutralization tests with Mengo encephalomyelitis and Uganda S virus antisera gave no evidence of any significant neutralization.

Discussion

The possible existence of minor antigenic relationships between Zika virus and other viruses has not yet been completely explored in this laboratory. From the evidence presented above, however, there is good evidence that Zika virus is not identical with any of the viruses against the immune sera of which it has been tested, and is not related to yellow fever, the Hawaii strain of dengue nor to the GD VII and FA strains of Theiler's virus. In addition to the serological tests which have been made, the pathogenicity and properties of Zika virus tend to differentiate it from other viruses, and it is believed that Zika virus is a hitherto undescribed virus.

The observations recorded in this paper are discussed in the paper which follows, along with studies on the pathogenicity and properties of Zika virus.

Summary

(1) The isolation of what is believed to be a hitherto unrecorded virus is described. The first isolation was made in April 1947 from the serum of a pyrexial rhesus monkey caged in the canopy of Zika Forest. The second isolation was made from a lot of *A. africanus* taken in January, 1948, in the same forest. The virus has been called Zika virus after the locality from where the isolations were made.

(2) Cross neutralization tests indicate that Zika virus is not related to yellow fever, Hawaii dengue nor to the FA and GD VII strains of Theiler's mouse encephalomyelitis virus. Neutralization tests with Zika virus and the antisera of some other viruses which are neurotropic in mice gave no evidence of any identity of these with Zika virus.

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