AN APPARENT INHIBITION PHENOMENON IN ANOPHELINE MOSQUITOES INFECTED WITH SINDBIS VIRUS

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Sindbis virus was first isolated from pools of *Culex* sp. mosquitoes in the region of Sindbis village, Egypt, by Taylor and Hurlbut (1953). Hurlbut (1953) was able to demonstrate the experimental transmission of Sindbis virus by *Culex pipiens* and *C. univittatus* mosquitoes to suckling mice. Collins and Harrison (1966) demonstrated the experimental transmission of this agent by *Anopheles albimanus* and *Aedes aegypti* mosquitoes to baby chicks.

During our previous studies, Sindbis virus titers in mosquitoes were individually determined immediately after taking their infectious blood meal and after various periods of extrinsic incubation. With the *An. albimanus* mosquitoes it was observed that, on some occasions, the titrations had abnormal mortality patterns in that the highest concentration of virus suspension resulted in very low mouse mortalities whereas high mortalities were obtained with more dilute concentrations. This did not occur with suspensions of infected *Ae. aegypti* mosquitoes, but a series of titrations with *An. stephensi* mosquitoes also showed an inhibition of mouse mortality in higher concentrations. This reaction appeared to be analogous to the pattern one sees as a prozone reaction in the complement fixation test, and further confirmation and possible explanation was desirable.

Reported here are the results of titrations of three species of mosquitoes infected with Sindbis virus, in two of which this phenomenon is demonstrated.

MATERIALS AND METHODS. The virus was Sindbis virus AR-1055, obtained through the courtesy of Dr. Phillip Coleman, National Communicable Disease Center, Atlanta, Georgia, U.S.A.

The *Ae. aegypti* were the CDC strain originally obtained from Technical Development Laboratories, CDC, Savannah, Georgia and maintained in our laboratory since 1959.

The *An. albimanus* mosquitoes were the A-9 strain originally obtained from San Salvador and maintained in our laboratory since 1960.

The *An. stephensi,* originally from India, were obtained from the London School of Hygiene and Tropical Medicine and have been maintained in our insectary since 1963.

Mosquitoes were infected by allowing

them to feed through a Baudruche (untreated) membrane on a virus suspension in heparinized rabbit blood. The virus suspension was prepared by grinding the brains of dead or moribund suckling mice previously inoculated with the seventh mouse passage of Sindbis virus in 20 percent chicken serum in Bacto-heart infusion broth (Difco) at the rate of one brain to 0.2 milliliter of diluent. After centrifugation for 15 minutes at 1,500 r.p.m., one milliliter of the supernatant was added to eight milliliters of freshly drawn heparinized rabbit blood.

For the mosquito feeding, the suspension was warmed to approximately 37° C. and placed on the membrane which formed the bottom of a ½ pint feeding cup. The cup was then placed on top of the cage containing the mosquitoes. The feeding period was 10 to 30 minutes, after which time the engorged mosquitoes were transferred to holding cages and stored in an incubator at 25° to 26° C. During the incubation period, the mosquitoes were fed 5 percent Karo solution daily on a cotton pledget.

Immediately after feeding and at various times during the incubation period, mosquitoes were killed by freezing, and stored in a mechanical freezer at —68° C. until titration. To determine the virus titers, mosquitoes were ground individually in a mortar with a one milliliter aliquot of broth to which 1,000 units of penicillin and two milligrams of streptomycin per milliliter had been added. The suspension was centrifuged for 15 minutes at 1,500 r.p.m. and serial 10-fold dilutions of the supernatant were made in the broth. Each dilution was used for the inoculation of one litter of 1- to 2-day old suckling mice (average of eight mice per litter). Each mouse was inoculated intracerabrally with 0.2 ml. of the dilution. The procedures and results of the transmission attempts are presented in the previous report (Collins and Harrison, 1966).

RESULTS. The results of the virus titrations obtained with the different species of mosquitoes are presented in Table 1. None of the titrations from five *Ae. aegypti* mosquitoes taken directly after feeding and none of the six taken 12 to 19 days later exhibited this inhibition. Of the six positive mosquitoes, three were able to transmit the infection by bite.

The titrations from 15 *An. albimanus* mosquitoes sampled at the time of infection all gave an expected pattern of mortality in that 100 percent of the mice died as a result of inoculation with the 10^{-1} and 10^{-2} dilutions of the mosquito-virus suspension. Atfer 12 days of incubation, however, titrations from eight mosquitoes resulted in mortality patterns in which the 10^{-1} dilution failed to kill any mice and the higheest percentage mortality was with the 10^{-3} dilution. Titrations from ten mosquitoes had expected patterns of response. A similar distribution occurred after 14 and 19 days of extrinsic incubation. Titration from 4 out of 12 and 2 out of 8 mosquitoes respectively showed inhibition with the more concentrated dilutions of the mosquito-virus suspension. For the period of 12 to 19 days, 14 out of 38 titrations (37 percent) demonstrated inhibition patterns. Two mosquitoes transmitted Sindbis virus and both of these failed to show the inhibition pattern upon titration.

Titrations from ten *An. stephensi* mosquitoes sampled immediately after infection all gave normal patterns of mortality in that 100 percent of the mice died when inoculated with the 10^{-1} and the 10^{-2} dilutions of the mosquito-virus suspension. All 32 titrations from mosquitoes sampled after 4 to 13 days of extrinsic incubation illustrated the inhibition pattern of mortality. No transmissions were attempted.

With the 46 titrations in which the inhibition effect was noted, the maximum percentage mortality was with the 10^{-3} dilution of the mosquito-virus suspension.

DISCUSSION. It would appear from these results that this inhibition effect is a common occurrence with two anopheline mosquitoes examined. Since transmissions

TABLE 1.—Percentage mortality in suckling mice inoculated with suspensions of triturated mosquitoes infected with Sindbis virus.

Mosquito species	Days post infection	Number of mosquitoes showing inhibition: Present	Absent	Percent mortality per dilution: 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Ae. aegypti	0	..	5	..	100	61	17	5	0
	12	..	3	100	100	88	74	27	10
	19	..	3	100	100	100	96	59	6
Total	12–19	..	6	100	100	93	83	45	7
An. albimanus	0	..	15	100	100	97	63	11	3
	12	8	..	0	8	67	18	3	..
	..	..	10	100	80	60	33	7	..
	14	4	..	19	52	75	54	18	..
	..	..	8	100	96	96	76	41	..
	19	2	..	0	10	70	25	0	..
	..	..	6	100	96	100	20	5	..
Total	12–19	14	..	5	20	69	27	7	..
	..	..	24	100	89	82	44	18	..
An. stephensi	0	..	10	100	100	82	40	4	1
	4	4	..	15	29	48	3	0	..
	6	4	..	19	28	68	36	..	..
	8	7	..	2	13	66	35	..	..
	10	4	..	6	3	70	26	10	..
	12	9	..	3	45	70	27	..	..
	13	4	..	9	62	76	26	..	..
Total	4–13	32	..	9	31	67	26	6	..

occurred only with those mosquitoes lacking the inhibition effect, its presence may in some way inhibit the transmission of this virus by an individual mosquito. The difference in distribution of this phenomenon among the three species of mosquitoes may suggest why one species is more capable of transmitting the infection than another. For example, three of six positive *Ae. aegypti* were able to transmit the virus whereas only 2 of 38 *An. albimanus* were capable of such transmission.

At present, the cause of this phenomenon is not clear. It can be postulated that since none of the 25 samples of *An. albimanus* and *An. stephensi* taken on day 0 illustrated this effect, it is not associated with the mosquito itself nor with the mouse-brain suspension containing the virus. It would appear that its presence is therefore associated with the incubation of the virus in the invertebrate host. The phenomenon whereby higher concentrations of virus result in lower mortality rates when inoculated into normally susceptible animals is commonly referred to as interference. It can be explained by the production of incomplete or non-infectious virus particles. Upon animal inoculation, these incomplete forms will not destroy the host cell, but will nonetheless block entry of the host cell by the infectious virus particles. However, upon dilution, fewer of these host cells are protected and the multiplying infectious virus particles are able to infect and destroy a sufficient number of cells to result in the death of the host. Another mode of action of the non-infectious virus particles could be the penetration of cells with stimulation of interferon production which could protect the majority of the cells against the live virus component of the inoculum. In the present study one would therefore suppose

that the *An. stephensi* and to some extent, the *An. albimanus* mosquitoes supported, in addition to the infectious forms, the production of a large amount of incomplete or non-infectious virus. The mosquitoes titrated immediately after feeding did not show this phenomenon since there had been insufficient time for the multiplication of the non-infectious virus.

Of interest is the possible application of this observation to the isolation of viruses from mosquitoes collected in the wild. The standard procedure as used by many workers, e.g. Kokernot *et al.*, 1957; Anderson *et al.*, 1957; Causey *et al.*, 1961; Groot *et al.*, 1961; and Dow *et al.*, 1964, is to triturate the mosquito or mosquitoes in a small amount of diluent and, after centrifugation, to inoculate the concentrated suspension intracerebrally and/or intraperitoneally into 1- to 2-day old mice. If such a procedure were used in attempts to isolate Sindbis virus from mosquitoes such as *An. albimanus* or *An. stephensi*, little or no mortality and therefore no apparent isolation would be obtained with the undiluted material. It would appear that an inoculation of the 10^{-3} dilution would more likely result in mortality and isolation if indeed the virus was present in the mosquitoes. Whether this phenomenon is applicable to other viruses or to mosquitoes other than anophelines is not at present known.

SUMMARY. The titration of mosquitoes infected with Sindbis virus resulted in mortality patterns in suckling mice indicative of an inhibition phenomenon. This response was not found in mosquitoes tested immediately after feeding but only after extrinsic incubation periods of 4 to 19 days. Its presence appeared to be more prevalent in *An. stephensi* than in *An. albimanus* and was not detected in *Ae. aegypti*.

It is postulated that the mosquitoes produce incomplete or non-infectious virus particles in response to infection with this virus. Upon inoculation into suckling mice, these non-infectious forms interfere with the invasion of the host cells by the infectious virus and thus the mortality of the mice. Its effect on the virus isolation procedures presently employed in arthropod-borne virus investigations is discussed.

Literature Cited

ANDERSON, C. R., AITKEN, T. H. G., DOWNS, W. G., and SPENCE, L., 1957. The isolation of St. Louis virus from Trinidad mosquitoes. Amer. J. Trop. Med. & Hyg. 6:688–692.

CAUSEY, O. R., CAUSEY, C. E., MAROJA, O. M., and MACEDO, D. G., 1961. The isolation of arthropod-borne viruses, including members of two hitherto undescribed serological groups, in the Amazon region of Brazil. Amer. J. Trop. Med. & Hyg. 10:227–249.

COLLINS, W. E., and HARRISON, A. J., 1966. Studies on Sindbis virus in *Anopheles albimanus* and *Aedes aegypti*. Mosq. News 26:91–93.

DOW, R. P., COLEMAN, P. H., MEADOWS, K. E., and WORK, T. H., 1964. Isolation of St. Louis encephalitis viruses from mosquitoes in the Tampa Bay area of Florida during the epidemic of 1962. Amer J. Trop. Med. & Hyg. 13:462–468.

GROOT, H., MORALES, A., and VIDALES, H., 1961. Virus isolations from forest mosquitoes in San Vicente de Chucuri, Colombia. Amer. J. Trop. Med. & Hyg. 10:397–402.

HURLBUT, H. S., 1953. The experimental transmission of a Coxsackie-like virus by mosquitoes. J. Egypt. Med. Assoc. 36:495–498.

KOKERNOT, R. H., HEYMANN, C. S., MUSPRATT, J., and WOLSTENHOLM, B., 1957. Isolation of Bunyamwera and Rift Valley fever viruses from mosquitoes. South African J. Med. Sci. 22:71–80.

TAYLOR, R. M., and HURLBUT, H. S., 1953. The isolation of Coxsackie-like viruses from mosquitoes. J. Egypt. Med. Assoc. 36:489–494.