EFFECT OF LOW ENVIRONMENTAL TEMPERATURE UPON JAPANESE B ENCEPHALITIS VIRUS MULTIPLICATION IN THE MOSQUITO [1]

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Recent information has indicated that the activity of certain arboviruses in nature is related to temperature (Hess *et al.*, 1963). One of the ways in which environmental temperature could affect virus activity is its influence upon virus multiplication in the poikilothermic vector, the mosquito. Several authors (Davis, 1932; Bates and Roca-Garcia, 1946; Chamberlain and Sudia, 1955; Rush, 1963) have demonstrated a relationship between the rate of virus multiplication and the temperature at which the infected mosquitoes were incubated. Other authors (Hurlbut, 1950; Bellamy *et al.*, 1958; Blanc and Caminopetros, 1930) have considered the effects of various temperatures upon viruses in the mosquito vector. The purpose of this study was to investigate the effect of a low temperature upon Japanese B encephalitis virus multiplication and the maintenance of virus titer in experimental mosquitoes. The possible effect on natural virus dissemination is discussed.

MATERIALS AND METHODS. The Japanese B encephalitis (JBE) virus used throughout this study was the third mouse brain passage of strain T-485, isolated from *Culex tritaeniorhynchus* by personnel of the 406th Medical General Laboratory in Tokyo, Japan. The *C. pipiens* colony was established from an egg raft collected in Frederick County, Maryland, and the *C. quinquefasciatus* colony from egg rafts obtained from the School of Hygiene and Public Health, the Johns Hopkins University. Virus titrations were made in weaned, eight- to ten-gram, 14-day-old albino Swiss-Webster mice, inoculated intracerebrally. The median lethal dose was calculated according to Reed and Muench (1938), and expressed as the logarithm of the number of mouse intracerebral median lethal doses contained in the examined tissue or stated volume of blood. "White Cross" chickens, the newly hatched progeny of a cross between a New Hampshire Red female and a male of 3:1 ratio, Vantress Red-Barred Rock, were used as transmission-indicator animals, and also served as a source of infective blood for the mosquitoes.

In order to infect a group of mosquitoes, a half-day-old chicken was inoculated subcutaneously with 300 mouse IC LD_{50} of JBE virus. Female mosquitoes were permitted to feed upon this chicken 72 hours later, and the virus concentration in the blood at the time of mosquito feeding was determined by titration in mice. Depending upon the purpose of the experiment, the mosquitoes were held for extrinsic incubation at 26.5° C. or, in certain instances, at other specified temperatures.

To determine the extent of virus multiplication in mosquitoes, or in parts of mosquitoes, a sample of from one to five individual mosquitoes was withdrawn from the cage at intervals after the infective blood meal. The entire mosquito or

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parts thereof were triturated individually in a mortar containing 1.0 ml. of diluent (10 percent rabbit serum-heart infusion broth with antibiotics). The resultant suspensions were centrifuged briefly to sediment large particles, serial 10-fold dilutions of the supernatant fluids were prepared in broth, and five mice were inoculated intracerebrally with each dilution. The titrations indicated the number of mouse IC LD_{50} per 0.03 ml. of the original 1.0 ml. of supernatant fluid, and did not measure any virus contained in the sediment. To calculate the virus contained in the total 1.0 ml. of supernatant fluid and thus arrive at an estimate of the total virus contained in the mosquito or part of the mosquito, the virus concentration per 0.03 ml. was multiplied by 33. Since the log value of 33 is approximately $10^{1.5}$, the lowest level of virus detection is indicated in the figures by a line at the $10^{1.5}$ LD_{50} level.

At intervals a larger sample of mosquitoes, usually about 25, was removed from the cage for a determination of the percentage infected and the percentage that could transmit the infection by bite to young chickens. Each mosquito was placed in a small plastic cylinder, which was then taped to the plucked dorsum of an uninfected half-day-old chicken. The 25 chickens were immobilized overnight to give the mosquitoes an opportunity to feed. The following morning the mosquitoes were triturated individually in diluent, and each suspension was tested for the presence of virus by mouse inoculation. The infection rate was defined as the percentage of mosquitoes that contained virus.

Each chicken upon which a mosquito had engorged was held for 72 hours, then bled from the heart. The blood was tested for virus by mouse inoculation. Viremia in a chicken was considered as evidence that its mosquito had transmitted the infection. The transmission rate was defined as the percentage of those mosquitoes known to have engorged which successfully transmitted virus to the chickens.

RESULTS. *Persistence of Infection in Mosquitoes Held at 10° C.* The work of previous authors (Blanc and Caminopetros, 1930) had suggested that exposure of dengue-infected mosquitoes to a cold environment might adversely affect the virus in some manner, a condition which could be rectified by a brief period of incubation of the mosquitoes at a warm temperature. To determine if exposure of infected mosquitoes to low temperature caused a reduction in JBE virus titer, *C. quinquefasciatus* females were infected as described above and incubated for 25 days at 26.5° C. (80° F.). At the end of this incubation period, 25 mosquitoes were removed from the cage as a sample, and the remaining individuals were placed in a cold chamber at 10° C. Each of the 25 mosquitoes in the 25th-day sample was found to contain virus and was able to transmit the infection successfully to the chick upon which it fed. It was therefore assumed that all the mosquitoes placed in the cold chamber had an active virus infection, and that all were now capable of transmission. At intervals of one to three days, a single mosquito was removed from the cold chamber, and after division into head, thorax, and abdomen, each part was separately assayed for virus concentration. Most of the mosquitoes tested throughout the observation period had virus titers between $10^{3.0}$ to $10^{5.9}$ mouse IC LD_{50}. There was no apparent loss of virus in the head, thorax, or abdomen (Fig. 1). On the 47th day, after a 22-day period of exposure to the cold, the remaining mosquitoes were used to determine a post-exposure infection rate and transmission rate. Twenty-three of 25 mosquitoes contained virus, and 8 of 11 that fed upon chickens transmitted it. These mosquitoes had virus titers ranging from $10^{1.9}$ to $10^{4.7}$ mouse IC LD_{50}.

In another experiment reported previously (LaMotte, 1958), *C. pipiens* were placed in the cold chamber after an incubation period sufficient to insure maximal involvement of salivary glands. Ten days later, a noninfected bat (*Eptesicus*

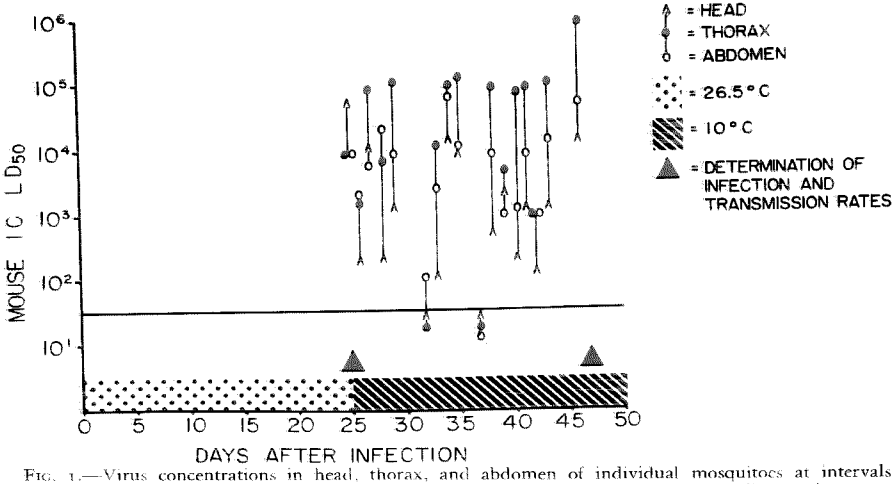

FIG. 1.—Virus concentrations in head, thorax, and abdomen of individual mosquitoes at intervals after exposure to 10° C. Transmission and infection rates were determined at the indicated time.

fuscus) was placed in the cage with the mosquitoes. Within two days, a single mosquito was visibly engorged with blood, and the bat was removed to a 24° C. environment. This bat, bled on the third day after removal from the cold, had $10^{2.4}$ LD_{50} of virus per 0.03 ml. of blood, indicating transmission of virus at 10° C.

Inhibition of Virus Multiplication in Mosquitoes at 10° C. Since the previous experiment indicated that virus persisted in mosquitoes held at 10° C, the next step was to determine whether virus multiplication was inhibited in mosquitoes held at this temperature. Approximately 400 *C. pipiens* were divided into two cages and designated as Groups A and B. The two groups were simultaneously infected from a single viremic chicken placed between the two cages. The mosquitoes received a calculated $10^{3.6}$ mouse IC LD_{50} of virus per 0.003 ml. in their blood meal. The Group A mosquitoes were held for three days at room temperature (24° C.), then at 26.5° C. for the duration of the experiment. At intervals after infection, individual mosquitoes were sacrificed; after division into head, thorax, and abdomen, each part was assayed for virus concentration (Fig. 2, Group A). The pattern of increasing virus titers was similar to that previously reported for mosquitoes held at 26.5° C. (LaMotte, 1960). On the 32nd day, the four Group A mosquitoes that remained successfully transmitted virus to chicks.

The Group B mosquitoes (Fig. 2) were also given a three-day period at room temperature in order to digest their infective blood meal, and were then placed in the cold chamber at 10° C. At intervals up to the 61st day, mosquitoes were removed from the cold chamber, usually one mosquito at a time, for assay of virus in head, thorax, and abdomen. Only 2 of 19 gave evidence of virus multiplication at this low temperature, and these specimens were so atypical as to suggest laboratory error.

As the sampling of Group B mosquitoes progressed with little evidence of the presence of virus, a test was made to determine if the Group B mosquitoes had lost their infection. About 70 mosquitoes were removed from the Group B cage to the warmer environment and designated as "Group B, first sample" (Fig. 2). Five of these mosquitoes were tested for the

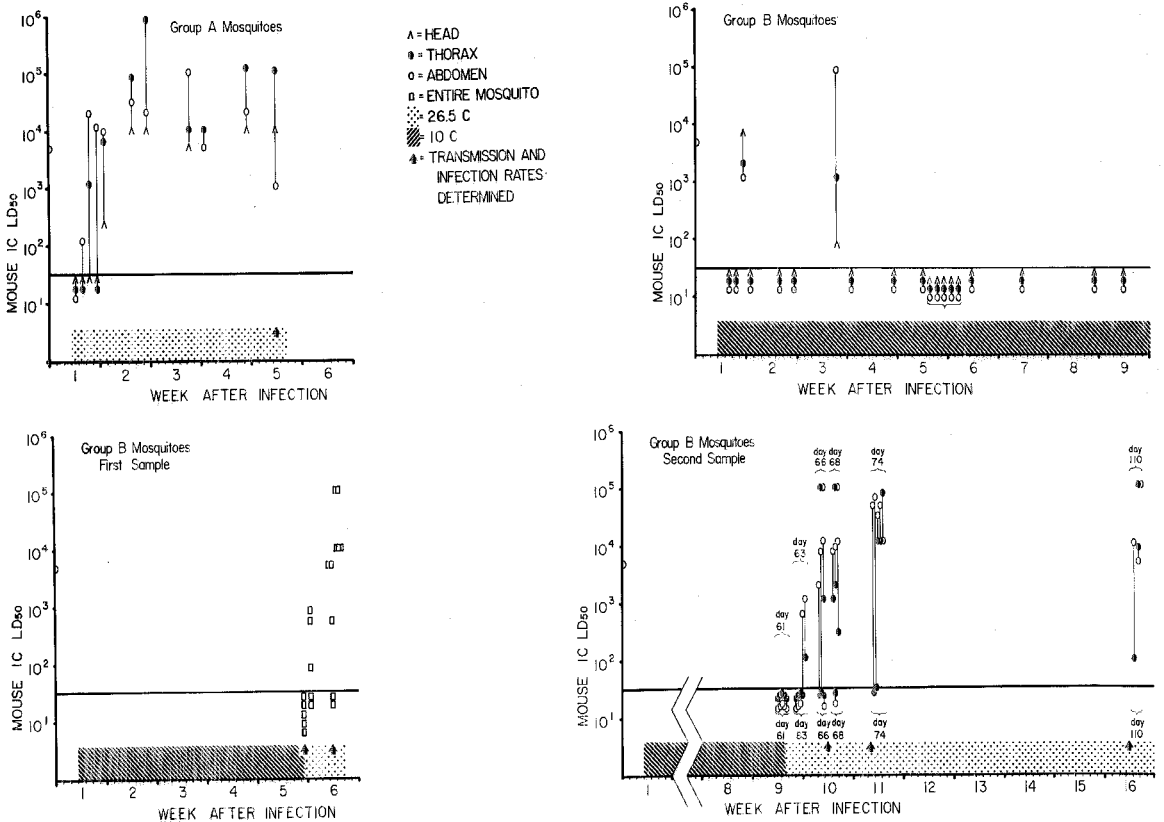

FIG. 2.—Virus concentrations in mosquitoes held at 26.5° C (Group A) and at 10° C (Group B), showing the effect of returning the Group B mosquitoes to a warmer environment (Group B, First and Second Sample).

amount of virus contained in the entire body of each mosquito immediately after removal from the cold, and further tests were made on five mosquitoes on the first, fourth, and fifth days of post-exposure incubation at 26.5° C. In addition, the transmission and infection rates were determined with 25 mosquitoes immediately after removal from the cold, and again with 25 more mosquitoes after they had been warmed for four days at 26.5° C.

The five mosquitoes tested immediately after removal from the cold had no detectable virus. After one day of incubation at 26.5° C, three of the five mosquitoes tested had detectable virus concentrations, and by the fifth day all five mosquitoes had 10,000 to 100,000 LD_{50}. Additional evidence of virus multiplication was obtained in the test to determine the transmission and infection rates. Of the 24 mosquitoes given an opportunity to feed upon chickens during the evening immediately following removal from the cold, only 10 took blood. None of these successfully transmitted the infection to the recipient chicks, although 21 of these mosquitoes had a detectable amount of virus when their suspensions were inoculated into mice the next day. However, there was frequently too little virulent virus in these mosquitoes to kill all five mice into which each mosquito suspension was inoculated.

In contrast, the mosquitoes held for a four-day period at 26.5° C. temperature before being tested gave evidence of a considerable increase in virus titer. Three of the 20 recipient chickens, which were fed upon by the mosquitoes, had demonstrable virus in their blood, indicating transmission by the three mosquitoes. Of the 25 mosquitoes tested for the presence of virus immediately after the transmission attempt, 22 had at least a sufficient amount of virus to kill all five mice into which each mosquito suspension was inoculated.

In view of the evidence that the Group B mosquitoes held at 10° C. had retained an infection, although with less than detectable virus concentrations, the remaining mosquitoes were removed from the cold on the 61st day. These mosquitoes, comprising the "Group B, second sample" in Figure 2, were held at 26.5° C. and the virus titers in the thorax and abdomen of sampled individuals were determined at intervals after removal from the cold. The five mosquitoes sampled immediately did not have detectable virus in either thorax or abdomen. After two days of incubation at 26.5° C. (day 63), virus was detected in two of five mosquitoes, and virus titers became increasingly higher in mosquitoes sampled on subsequent days. Although very few mosquitoes remained for transmission attempts, three mosquitoes were each fed on a separate chicken on the sixth day after removal from the cold (day 67), but did not transmit virus; after 12 days of post-exposure incubation, one of five mosquitoes transmitted the infection to a chicken; after 48 days at the warmer temperature two of three mosquitoes successfully transmitted the infection. Each of the 11 mosquitoes used in the transmission attempts contained virus.

DISCUSSION. The temperature dependence of JBE virus multiplication in the poikilothermic mosquito suggests that the environmental temperature in nature could materially affect the natural history of this arbovirus. A relationship has been observed between spring temperatures and the extent of WE and SLE activity (Hess *et al.*, 1963). Further, outbreaks and enzootic activity of St. Louis encephalitis virus appear to be limited to the warmer, southern areas of the United States *(ibid.)*, and JBE, a closely related virus, is usually similarly limited in Japan (Warren, 1946).

The data indicate that once JBE infection is firmly established in the mosquitoes, temporary subjection to cold temperatures will not cause sufficient virus loss in the infected mosquitoes to change radically their infection rate or their potential for transmitting the virus with which they are infected. If sufficiently low temperature conditions are imposed soon after the mosquitoes have

taken an infective blood meal, virus multiplication will not occur to a significant degree until the temperature again rises. Infected mosquitoes showed no evidence of having cleared their system of virus; on the contrary, their bodies either do not recognize virus as foreign protein or have no efficient antibody-producing tissues. The arboviruses apparently find a hospitable milieu in the tissues of the mosquito, and the accumulated evidence indicates that once a mosquito is infected it remains infected for life.

When freshly engorged mosquitoes were subjected to the cold, it was noted that blood meal digestion stopped or slowed to an imperceptible rate, as had virus multiplication in infected mosquitoes. The primary site of virus multiplication is thought to be in the midgut epithelial cells (LaMotte, 1960), and digestion is a function of these same cells. It seems probable that low temperature affects the metabolic rate of the midgut cells and thereby minimizes both virus multiplication and digestion.

Under natural conditions, an occasional cold period with late afternoon temperatures below a critical point might be expected to delay temporarily the build-up of virus in a bird-mosquito cycle by inhibiting virus multiplication in the mosquito population. A more protracted period of cold might affect adversely the production of mosquitoes, and, therefore, affect the bird-mosquito virus cycle which is responsible for extensive virus dissemination.

SUMMARY. Japanese B encephalitis virus multiplication in experimental mosquitoes was inhibited when the mosquitoes were held at 10° C. Virus infection persisted in these mosquitoes, as indicated by the rapid virus multiplication when they were returned to 26.5° C. Low temperature did not appear to affect adversely the virus titer attained in the mosquitoes prior to exposure to the cold, at least during the observation period reported. It is suggested that low temperatures in nature could exert an effect upon JBE virus transmission by inhibiting virus multiplication in the mosquito vector.

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