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13. I thank L. Bravard and D. Schoch for technical assistance. Supported in part by NIH grants EY00860 and EY01171.

28 June 1973; revised 3 September 1973

Transovarial Transmission of LaCrosse Virus (California Encephalitis Group) in the Mosquito, Aedes triseriatus

Abstract. *LaCrosse virus (California encephalitis group) was recovered from F_1 eggs, larvae, and adults produced by experimentally infected* Aedes triseriatus. *The F_1 females transmitted the virus by bite to suckling mice and chipmunks. This, plus isolations of LaCrosse virus from larvae collected from their natural habitats in enzootic areas and from males and females reared from them, suggests that transovarial transmission is the overwintering mechanism for this arbovirus in northern United States.*

Epidemiological studies in Wisconsin have indicated that LaCrosse (LAC) virus is maintained in nature by an *Aedes triseriatus*–small mammal cycle (*1*). *Aedes triseriatus* has proved to be a very efficient laboratory transmitter of LAC virus (*2*) and considerably more efficient than nine other species of mosquitoes (*3*).

The method of survival of LAC virus during the winter months when adult mosquitoes are inactive has remained unknown; however, isolations of the virus from *A. triseriatus* larvae (*4*) in 1972 suggested overwintering of LAC virus in diapause eggs laid by *A. triseriatus*. In view of the implication of *A. triseriatus* as the major vector of LAC virus and the isolations from *A. triseriatus* larvae, this investigation was conducted to explore the possibility of transovarial transmission of LAC virus in this mosquito. In our experiments LAC virus was recovered from F_1 eggs, larvae, and adults produced by experimentally infected *A. triseriatus*. The F_1 females transmitted the virus by bite to suckling mice and chipmunks.

The LAC virus used was originally isolated from human brain tissue (*5*). It had undergone four serial intracerebral passages in suckling mice, one subcutaneous passage in chipmunks, and a fifth intracerebral passage in suckling mice.

Aedes triseriatus mosquitoes, 7 to 14 days of age and in their fifth generation of colonization, ingested LAC virus in guinea pig defibrinated blood. The virus content of the infectious bloodmeal was determined by the intracerebral inoculation into suckling mice of serial tenfold dilutions of the blood-virus mixture upon which the mosquitoes had fed. As a matter of convenience, control mosquitoes were fed on hamsters rather than through a membrane. Portions of blood from each animal were inoculated intracerebrally into suckling mice to show that mouse lethal agents were not present.

New engorged mosquitoes were transferred to screen cages or to ice-cream (about 500-ml size) containers. An oviposition site consisting of a moist strip of black cotton cloth inserted along the inner wall of a crystallizing dish (50 by 35 mm) was placed in each cage or container. A 5 percent solution of sucrose in water was provided the mosquitoes during the extrinsic incubation period. All mosquitoes, including developmental stages, were maintained at 27°C and 80 percent relative humidity under a 16-hour photoperiod. Controls, including eggs, larvae, and adult mosquitoes were maintained under similar environmental conditions. The possibility of inadvertent introduction of virus in the experiments was prevented by (i) maintaining the mosquitoes in an insectary in which no other experimental studies with LAC virus or other members of the California virus group were being conducted, (ii) sterilizing equipment used in maintaining mosquitoes, (iii) assaying specimens in a separate laboratory in which there had been no previous work with known arboviruses, and (iv) removing brain tissue from mice in a room in which there had been no previous work with viruses.

At various intervals during the extrinsic incubation period parent females were allowed to feed on adult hamsters or 1- to 3-day-old suckling mice, one mosquito per animal. This provided blood for egg development as well as providing an indicator of infection in parent mosquitoes. Immediately after each feeding the oviposition site was removed and replaced with a new one. After completing several ovarian cycles, mosquitoes were placed at −70°C for subsequent testing for the presence of virus. Approximately 25 percent of the eggs from each ovarian cycle were removed for virus assay while the remaining eggs were inundated in a nutrient solution of broth and water (1 : 1000). The first-instar larvae were transferred to tap water to which Tetramint was added as a food source. When larvae developed to the third or fourth instar, a pool of ten larvae from each ovarian cycle was stored at −70°C for virus assay. The F_1 females were retained for 7 to 14 days before they were allowed to feed on suckling mice. After feeding on mice, mosquitoes were placed at −70°C or retained for feeding on chipmunks, one mosquito per animal. Serial tenfold dilutions of blood specimens taken from the orbital sinus of each chipmunk were assayed for virus by a plaque test (*6*).

Adult mosquitoes were triturated individually in the U-shaped cavity of an 11-mm, sterile, rubber-style vaccine bottle stopper with a 1.0-ml glass syringe plunger. Eggs and larvae were triturated similarly, the eggs in pools of varying numbers and the larvae in pools of ten. Triturated specimens were suspended in 1.0 ml of medium similar to that employed for making dilutions of the infectious bloodmeal. Suspensions were assayed for virus by intracerebral inoculation of 1- to 3-day-old mice, 0.03 ml per mouse. All mice were observed for 10 days. The brains of moribund and dead mice were removed aseptically for use in neutralization tests. Recovery of LAC virus from mosquito suspensions, from brain tissue of mice, and from blood of chipmunks was confirmed by neutralization tests with the use of LAC hyperimmune mouse ascitic

fluids prepared according to the technique of Brandt (*7*). Virus infectivity endpoints were calculated by the method of Reed and Muench (*8*).

Vertical transmission of LAC virus was first observed in progeny originating from 30 female mosquitoes. These parent mosquitoes all became infected after ingesting an infectious bloodmeal containing $10^{6.4}$ $SMLD_{50}$ (suckling mouse lethal dose, 50 percent effective) per 0.03 ml of blood. Most of the mosquitoes survived for 43 days during which time they engorged on hamsters or suckling mice at 10- to 12-day intervals. The eggs laid after each bloodmeal were considered to represent the first, second, third, and fourth ovarian cycle, respectively.

Eggs from the first ovarian cycle were not tested for virus, and virus was not recovered from a pool of 35 eggs representing the second ovarian cycle. Virus was recovered from two pools, each containing 75 eggs, representing the third and fourth ovarian cycles. The concentration of virus appeared to be very low because only three of six mice died after inoculation of either positive pool.

LAC virus was recovered from larvae that hatched from eggs representing each of the four ovarian cycles. Each larval suspension on inoculation into suckling mice produced 100 percent mortality, suggesting an increased virus concentration in larvae compared to that in the eggs.

LAC virus was recovered from adult mosquitoes of both sexes originating from each ovarian cycle. Of 177 F_1 mosquitoes tested, virus was recovered from 33 of 85 males and from 28 of 92 females. When 63 of the 92 females fed individually on suckling mice, 21 mosquitoes were capable of transmitting a lethal dose of virus. The recovery of virus from larvae and F_1 adults originating from eggs of ovarian cycle 2 from which virus was not recovered indicates that the eggs contained either a nondetectable level of virus or virus in a nondetectable (eclipsed) state.

No virus was detected in controls consisting of 110 parent mosquitoes that ingested normal hamster blood and 900 eggs, 100 larvae, and 100 F_1 adults of these parent mosquitoes. Blood samples taken from hamster and guinea pigs were shown to be free of mouse lethal agents.

In the second experiment, *A. triseriatus* mosquitoes ingested an infectious bloodmeal containing $10^{5.0}$ $SMLD_{50}$ per 0.03 ml of blood. After the first ovarian cycle was completed, the mosquitoes were allowed to feed individually on suckling mice and then transferred to ice-cream containers (500-ml size), one mosquito per container. Of 11 batches of second ovarian cycle eggs laid by 11 infected females, virus was recovered from a pool of 19 eggs from one mosquito. This mosquito was the only one that laid fertile eggs. Virus was detected in a pool of ten larvae hatching from eggs of this female and in the emerging adults, including all of five males and all of four females. Each of the four females transmitted virus to suckling mice 8 days after emergence. Two of these mosquitoes fed on chipmunks 5 days later, and one of the two mosquitoes became infected as indicated by a viremia that was detected on days 2, 3, and 4 after feeding. The same two animals fed individually on two more chipmunks 12 days later, or 25 days after ingesting the infectious bloodmeal. A viremia was observed in both animals on days 2, 3, and 4 after feeding. Viremia ranged from $10^{0.5}$ to $10^{3.7}$ plaque-forming units per 0.1 ml, which is well within the concentration required to infect *A. triseriatus* mosquitoes (*2*).

Since vertical transmission of LAC virus in *A. triseriatus* could possibly have occurred by a route (or routes) other than transovarial, an experiment was conducted to determine whether virus was on the surface or inside the eggs. First, eggs laid by uninfected *A. triseriatus* were submerged in $10^{6.0}$ $SMLD_{50}$ per 0.03 ml of LAC virus for 16 hours at 4°C. The eggs were then divided into two groups, 250 eggs per group. One group received no treatment, while the other was rinsed for 10 minutes with a solution of 1.0 percent Chlorox in distilled water followed by a 10-minute rinse with 70 percent alcohol and a 3-minute rinse with sterile distilled water. Each group of eggs was triturated, and, on intracerebral inoculation into suckling mice, LAC virus was recovered only from the untreated group of eggs. LAC virus was not recovered from adult mosquitoes originating from eggs of either group, indicating that transmission by surface contamination of eggs did not occur. The above procedure was used to treat eggs laid by a LAC-infected mosquito. LAC virus was recovered from both the treated and untreated group of eggs, and six of eight F_1 female mosquitoes originating from surface-sterilized eggs transmitted virus by bite to suckling mice, thus indicating that the virus was inside the egg.

The above findings, together with the isolations of LAC virus from field-collected larvae (*4*) constitute the first conclusive demonstration of transovarial transmission of an arbovirus by mosquitoes. That this is the overwintering mechanism of LAC virus is further supported by (*9*): (i) additional isolations of LAC virus on several occasions from *A. triseriatus* larvae collected from tree holes and from a discarded automobile tire during April, May, and June of 1973 prior to the emergence of adult *A. triseriatus*; (ii) the isolation of LAC virus from female *A. triseriatus* originating from larvae collected from the tires and the transmission of virus to suckling mice by these mosquitoes; (iii) the isolation of the LAC virus from male *A. triseriatus* originating from larvae collected from water of tires and basal tree holes.

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10. Supported in part by NIH grants AI-07453 and AI-00771. We thank D. Fischer for technical assistance.

3 August 1973; revised 7 September 1973