Venereal Transmission of La Crosse (California Encephalitis) Arbovirus in *Aedes triseriatus* Mosquitoes

Abstract. *Venereal transmission of La Crosse virus by males of* Aedes triseriatus *was demonstrated. La Crosse virus was detected in the bursa of females after induced copulation, and disseminated infection was shown to occur occasionally. Since males of* Aedes triseriatus *have transovarial filial infection rates similar to those of females and can repeatedly mate, venereal transmission may be an important supplement to other natural endemic maintenance mechanisms.*

We demonstrated previously that horizontal transmission of La Crosse (LAC) arbovirus from male to female *Aedes triseriatus* occurs in mating cages (*1*). This, combined with the demonstration of large amounts of viral antigen in reproductive tract tissues of the mosquitoes, especially in the male accessory sex organs (*2*), prompted our investigation of possible venereal transmission of the virus.

Since male and female *A. triseriatus* had similar filial infection rates (*3*) with LAC virus upon emergence as adults from sites of larval development (tree holes), venereal transmission was considered a potentially important supplement to transovarial transmission (*4*) and infection by feeding on viremic vertebrates (chipmunks and squirrels) (*5*) for the natural maintenance of this arbovirus which causes encephalitis in children.

Two strains of female *A. triseriatus* mosquitoes were used. One was a long-established laboratory colony, the other was the F_2 generation of a colony (ZN) established from larvae collected from a basal tree hole near La Crosse, Wisconsin, during 1975. These colonies were demonstrated to be free of LAC virus during repeated use in other studies, by the fluorescent antibody (FA) technique, and by isolation tests of parent stocks. The LAC virus used was the prototype, human isolate (*6*) at the fourth passage in suckling mouse brain.

To preclude modes of horizontal virus transmission other than venereal, the limited contact technique of induced insemination (*7*) was used for mating. Females were anesthetized in a nitrogen bath according to methods suggested by Grimstad (*8*), and the sexual organs only were brought into contact, typically for 20 to 80 seconds.

Males were infected with LAC virus (approximately 10 to 100 LD_{50}, where LD_{50} is the lethal dose for 50 percent of the mouse population, in *p*H-buffered diluent containing antibiotics; 0.00006 ml per mosquito) by intrathoracic inoculation (*9*) 12 or more days prior to mating. By means of the FA technique, LAC antigen was routinely observed in many tissues, including the accessory sex glands of males dissected soon after induced copulation.

After mating, the females were held individually in cartons in an insectary maintained at 80 percent relative humidity, and 25°C, and were subjected to a photoperiod of 16 hours of light and 8 hours of darkness. Three percent sucrose solution was freely available.

After incubation periods ranging from 10 minutes to 14 days after mating, female abdomens were dissected and processed according to the FA technique for demonstration of the LAC antigen in various organs as previously reported. Heads, thoraxes, and other remnant portions were individually stored frozen for later attempts to isolate the virus.

Venereal transmission of LAC virus by inoculated males was proved by the demonstration of LAC antigen in the bursal contents or in the mating plugs of the females soon after induced insemination, and later by demonstration in some of the females of antigen in tissues other than those of the lower reproductive tract.

The percentages of females showing antigen in the bursal contents decreased progressively with time. At 10 minutes after insemination, 100 percent (17 out of 17 dissected) of the females showed LAC antigen; the cumulative number of females showing antigen decreased to 79 percent (23 out of 29) by 6 hours, 76 percent (26 out of 34) by 18 hours, 53 percent (35 out of 66) by 24 hours, 46 percent (36 out of 78) by 48 hours, 44 percent (40 out of 91) by 3 days, and 24 percent (42 out of 172) by the end of 6 days (Table 1). No antigen was observed in bursal contents of 188 females dissected 7 to 14 days after induced mating.

Disseminated infections (into organs other than those of the lower reproductive tract) were detected in 3.4 percent (13 out of 379 dissected) of venereally infected females dissected up to 14 days after insemination. The earliest disseminated infection was noted on day 3 in 0.9 percent (1 out of 109). It was detected in 3.7 percent (3 out of 82) on days 4 to 6, 4.6 percent (3 out of 66) on days 7 to 9, and in 4.9 percent (6 out of 122) on days 10 to 14 (Table 1).

Transmission of LAC virus to a mouse was demonstrated by the isolation of LAC virus from the brain of one of eight mice bitten by venereally infected females held for 30 days after insemination.

Isolations of LAC virus were obtained by intracranial inoculation into suckling mice of (i) remnants of males in which antigen had been visualized in accessory sex glands, (ii) pools of bursa from females dissected 24 hours after induced insemination, and (iii) remnants from six of the 13 females in which viral antigen had been later observed by FA in organs other than those of the lower reproductive tract.

Venereal transmission by inoculated males was demonstrated in females from both previously negative colony sources. Transovarially infected males were also shown capable of venereally infecting females. Multiple transmission of LAC to

Table 1. Observation of the LAC antigen by means of the FA technique in various organs of female *A. triseriatus* dissected at intervals after induced insemination by males previously infected by inoculation. All of these inoculated males were from the colony ZN established from larvae collected in 1975; they were infected with LAC virus by intrathoracic inoculation 15 and 16 days prior to induced insemination and had observable antigen in accessory sex glands when dissected. Females were from the colony ZN and from the long-established laboratory (LAB) colony.

Female source	Time of dissection after induced insemination					
	0 to 6 days		7 to 14 days		Total 0 to 14 days	
	Ratio	%	Ratio	%	Ratio	%
In bursal contents or mating plugs						
ZN	24/86	28	0/103	0	24/189	12.7
LAB	18/86	21	0/ 85	0	18/171	10.5
ZN and LAB	42/172*	24	0/188*	0	42/360*	11.7
In organs other than the lower reproductive tract						
ZN	2/98	2	7/103	7	9/201	4.5
LAB	2/93	2	2/ 85	2	4/178	2.3
ZN and LAB from both colonies	4/191†	2	9/188†	5	13/379†	3.4

*Ratio of the number of females with LAC antigen observed in bursal contents to the number examined. †Ratio of the number of females with antigen in organs other than the lower reproductive tract to the number examined.

virus-free females by infected males was demonstrated by the finding of LAC antigen in the bursa of one female and a disseminated infection in another mated to the same male.

By means of the FA technique, LAC viral antigen was detected in large amounts in the accessory sex glands of males, accessory gland fluids extruded from the ejaculatory duct, bursal contents, and mating plugs from recently mated females from the two previously LAC virus-free colonies, and in organs other than the lower reproductive tract (including heart, nerve ganglia, ovaries, gut, salivary glands, or fat bodies) of some of these females.

In infected males, antigen was also observed by FA in the testes and seminal vesicles, but in amounts much lower than those found in accessory sex gland fluids. Antigen was not observed within sperm. It would seem that LAC virus in *A. triseriatus* is venereally transmitted by male accessory sex gland fluid, which agrees with findings of other investigations with other vector-pathogen systems (*10*).

Additional knowledge of the natural cycle of LAC arbovirus is essential because of its growing public health importance and the difficulty of controlling the vector species (which survives in protected tree-hole sites) by conventional methods utilizing chemical insecticides. The frequency and importance of venereal transmission in nature are yet to be ascertained. However, such transmission may be an important supplement to transovarial and viremic mammalian blood virus sources for the natural endemic maintenance of this arbovirus.

WAYNE H. THOMPSON
BARRY J. BEATY*
Zoonoses Research Unit, Department of Preventive Medicine, University of Wisconsin, Madison 53706

References and Notes

1. W. H. Thompson, B. J. Beaty, R. Anslow, paper presented at the annual meeting of the American Society of Tropical Medicine and Hygiene, New Orleans, 10 November 1975.
2. B. J. Beaty and W. H. Thompson, *Am. J. Trop. Med. Hyg.* **25**, 505 (1976).
3. ———, *ibid.* **24**, 685 (1975).
4. D. M. Watts, S. Pantuwatana, G. R. DeFoliart, T. M. Yuill, W. H. Thompson, *Science* **182**, 1140 (1973).
5. D. W. Moulton and W. H. Thompson, *Am. J. Trop. Med. Hyg.* **20**, 474 (1971).
6. W. H. Thompson, B. Kalfayan, R. O. Anslow, *Am. J. Epidemiol.* **81**, 245 (1965).
7. W. R. Horsfall, H. W. Fowler, L. V. Morette, Jr., J. R. Larson, *Bionomics and Embryology of the Inland Mosquito* Aedes vexans (Univ. of Illinois Press, Chicago, 1973).
8. P. Grimstad, personal communication.
9. L. Rosen and D. Gubler, *Am. J. Trop. Med. Hyg.* **23**, 1153 (1974).
10. W. Burgdorfer and G. R. Varma, *Annu. Rev. Entomol.* **12**, 347 (1976); W. Plowright, C. T. Perry, A. Greig, *Res. Vet. Sci.* **17**, 106 (1974); P. E. Fine, *Ann. N.Y. Acad. Sci.* **266**, 173 (1975); A. Spielman, *ibid.*, p. 115.
11. We thank J. Schallern, C. Johnson, and L. Lorenz for technical assistance. Supported by PHS research grant 2 RO1-AI-11547 from the National Institutes of Health.
* Present address: Yale Arbovirus Research Unit, Yale University, New Haven, Conn. 06510.

2 September 1976; revised 15 November 1976

Internal Calcium Changes in a Bursting Pacemaker Neuron Measured with Arsenazo III

Abstract. *Arsenazo III was used to measure changes in the free intracellular calcium ion concentration during spontaneous bursting pacemaker activity in the* Aplysia *R15 neuron. Intracellular calcium increased during the burst, and this increase was sufficient to cause the hyperpolarization that followed. The results suggest that the interval between bursts is determined by the rate of subsequent decline of free intracellular calcium.*

One of the characteristic features of excitable cells is the ability to generate rhythmic activity. Certain neurons share with heart and smooth muscle cells the capability of spontaneously generating rhythmic patterns of action potentials, which are used to control or drive other cells. The origin of this behavior is still not fully understood. The neuron R15 (*1*) in the abdominal ganglion of the mollusk *Aplysia californica* is of this type (*2*) and has been extensively studied. Particular attention has been paid to the mechanism responsible for the hyperpolarization that occurs after each spontaneous burst of action potentials (*3–6*). Action potentials in *Aplysia* neurons are abolished by Ca^{2+}- and Na^+-free saline but not by Na^+-free saline alone (*7*), which suggests that a significant proportion of the inward current is carried by Ca^{2+} ions. A Ca^{2+} influx during the action potential has been directly shown with the Ca^{2+}-sensitive photoprotein aequorin (*8*). Injection of Ca^{2+} into *Aplysia* neurons causes a hyperpolarization, which results from an increase in K^+ conductance (*9*). It has been shown that the hyperpolarization that follows each spontaneously generated burst of action potentials is accompanied by an increase in K^+ conductance (*4, 6*). The possibility that the Ca^{2+} influx during a burst of action potentials may be sufficient to increase the K^+ conductance is strengthened by the demonstration of a posttetanic hyperpolarization in R15, following a train of action potentials induced by membrane depolarization. The effect is abolished by Ca^{2+}-free saline or injection of [ethylenebis (oxyethylenenitrilo)] tetraacetic acid (EGTA), which suggests that it is mediated by an increase in free intracellular Ca^{2+} (*5*). It is conceivable, therefore, that free intracellular Ca^{2+} may increase during each train of action potentials and be an important determinant of the bursting behavior by causing, or assisting in causing, the hyperpolarization that follows.

To test this theory, it is necessary to determine directly whether free intracellular Ca^{2+} changes and to measure the size and time course of these changes throughout the burst cycle. The Ca^{2+}-sensitive dye arsenazo III, which undergoes an absorbance change on forming a complex with Ca^{2+} (*10*), has been used successfully to detect changes in free intracellular Ca^{2+} (*11, 12*). We used this dye to follow such changes during the burst cycle in R15, and we report here the results of these experiments.

The abdominal ganglion was dissected to expose R15, and a fiber-optic probe was positioned on each side of the neuron. Light from a tungsten halide source was passed through a wheel driven at 12,000 rev/min by compressed air; the wheel contained several narrow-band interference filters of different wavelengths, which sequentially interrupted the light beam. The composite light signal that resulted was directed to R15 through one of the fiber-optic probes, and the fraction that passed through the cell was directed to a photodiode through the second probe. The output from the photodiode was electronically separated into the components that corresponded to each filter wavelength for subsequent manipulation. The dye was injected under pressure through an intracellular microelectrode to give an internal concentration (as judged by the absorbance increase) of approximately 0.3 m*M*. This concentration did not affect the electrical activity of the cell. One or more additional electrodes were inserted for injection of ions and electrical recording. Changes in dye absorbance were measured by differential recording between a wavelength at which Ca^{2+} caused no absorbance change (570 nm) and one at which the absorbance increase was maximal (650 nm). The experiments were performed in artificial seawater (*13*) at 16°C.

A typical result, shown in Fig. 1, provides strong evidence for an increase in the free internal Ca^{2+} concentration during the burst. The absorbance changes as-