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STUDIES ON SELECTION OF LACROSSE VIRUS VARIANTS BY NATURAL VERTEBRATE HOSTS AND VECTOR MOSQUITOES

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Summary. — The effect of passage in natural hosts on LaCrosse virus plaque size, mouse neurovirulence, and plaquing efficiency was studied. Seven field strains, as previously unpassaged naturally infected *Aedes triseriatus* suspensions, were inoculated into chipmunks *Tamias striatus* and thence into colonized *Ae. triseriatus*. The prototype strain, with seven prior laboratory passages, was also passed through the same natural host species. The field strains changed in neurovirulence and plaque size at various passage levels, but with no consistent pattern. The prototype strain was relatively unvarying in plaque size, but changed moderately in neurovirulence. For all eight strains, chipmunk passage reduced plaquing efficiency beyond the nonspecific inhibition normally found in chipmunk blood. We conclude that natural hosts may select variants in unpredictable patterns from highly variable unpassaged field strains, but that prior laboratory passage reduced the prototype strain to a relatively homogeneous population resistant to change in at least one aspect.

Key words: Bunyaviridae; plaque variants; virulence variants; plaquing efficiency; vertebrate host; arthropod vector

Introduction

Arbovirus populations are variable, and consist of subpopulations with different biological properties. Within the California group of *Bunyaviridae*, variation in plaque size, neurovirulence, and temperature sensitivity has been studied for Ťahyňa virus (Málková, 1971, 1974; Málková and Reddy, 1975), and Jamestown Canyon and LaCrosse (LAC) viruses (Issel *et al.*, 1975; Ksiazek and Yuill, 1977*a*; Hansen, 1976). These studies have shown that passage in cell cultures and laboratory mice can select for certain variants. Similar variant selection by passage of a pathogen in wild hosts in natural cycles might be epidemiologically significant.

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Table 1. Inoculation of chipmunks Tamias striatus with eight strains of LaCrosse virus

Virus strain	Dose*	No. of animals inoculated
77—88	2.2	1
77—92	3.9	1
77—124	1.9	1
77—151	<1.5	1
76—22	3.6	2
76—26	Not tested	2
76—40	2.0	2
Prototype	3.0	1

* $\log_{10}$ SMicLD$_{50}$ per total inoculum of 0.2 ml.

The natural hosts of LAC virus, a cause of human disease in the United States, include chipmunks (*Tamias striatus*) and gray squirrels (*Sciurus carolinensis*) (Moulton and Thompson, 1971; Pantuwatana *et al.*, 1972; Gauld *et al.*, 1974, 1975; Ksiazek and Yuill, 1977*b*). Sequential laboratory passages of LAC virus in these animals may select for plaque size variants (Issel *et al.*, 1975) and increase squirrel infectivity (Hansen, 1976). Low-passage LAC strains isolated from chipmunks and squirrels vary in plaque size and mouse virulence (Ksiazek and Yuill, 1977*a*). However, all of these studies of LAC virus variants in natural hosts have used virus strains passed at least once in mice, a procedure known to select for small plaque variants (Ksiazek and Yuill, 1977*a*).

The purpose of this study was determine if characteristics of unpassaged LAC virus field isolates were changed by alternate passage through a natural vertebrate host, the chipmunk, and the main arthropod vector mosquito (*Aedes triseriatus*). The characteristics examined were mouse neurovirulence, plaque size, and plaquing efficiency.

Materials and Methods

Viruses. Seven field strains of LAC virus were received from Dr. Wayne Thompson, Department of Preventive Medicine, University of Wisconsin, Madison. They originated from naturally transovarially infected *Ae. triseriatus* larvae collected from tree holes in the LAC virus enzootic area of southwestern Wisconsin. These seven strains were used as larvae suspensions without laboratory passage. For comparison, the prototype LAC virus strain was used after six passages in mouse brain and one in Vero cells.

Experimental animals. Wild chipmunks trapped near Madison, Wisconsin were tested by microneutralization test (Sukhavachana *et al.*, 1969) for serum neutralizing antibody against LAC, snowshoe hare, trivittatus, and Jamestown Canyon viruses, the four California group agents known to occur in Wisconsin. Animals whose sera did not react at 1 : 5 against any of these viruses were used in experiments. Chipmunks were inoculated as adults, at least three months old. Colonized *Ae. triseriatus* mosquitoes of the fifth and sixth generation from the wild were donated by Dr. Gene DeFoliart, Department of Entomology, University of Wisconsin-Madison and maintained in his laboratory at 25 ± 2 °C and 80 ± 5% relative humidity.

Virus titration and neurovirulence assays in mice. We routinely inoculated 0—1 day old mice (SM) intracerebrally (i. c.), for estimation of virus titers (SMicLD$_{50}$) according to Reed and Muench. We studied neurovirulence by the method of Málková (1971), inoculating 8—9 day old

mice with 0.025 ml i. c. or 0.1 ml subcutaneously (s. c.). The neurovirulence index is the ratio of the i. c. titer divided by the s. c. titer, adjusted for equal volumes; the index increases with decreasing neurovirulence. We confirmed LAC virus in the brains of sick or dead mice by microneutralization tests using reference prototype LAC virus hyperimmune mouse ascitic fluid.

Plaque assay and plaque size determination. We used Vero cells obtained from the Gorgas Memorial Laboratory, Panama, after 144—164 passages from the original African green monkey kidney. In most tests, plaquing procedures followed Issel *et al.* (1975) for tests with crystal violet staining. Other tests were similar, but used an overlay of 0.4% gum tragacanth, 0.5% Sigma II agarose and 5% fetal calf serum in Eagle's minimal essential medium. After four days incubation at 37 °C in 5% CO_2, an equal volume of the same overlay containing 0.015% neutral red was added; plaque-forming units (PFU) could be read after 24 hr of further incubation.

We used the technique of Ksiazek and Yuill (1977*a*) to measure plaque diameters, and included an indentical stock prototype virus standard in each test to control for test-to-test variability. This prototype reagent contained two distinct plaque sizes by inspection. The minimum diameter of the large size class was almost always twice the overall median diameter, and the maximum size of the apparently small plaques was almost without exception smaller than twice the overall median diameter. The mean diameter of the small prototype control plaques, as defined by this empirical statistic, was divided into each simultaneously-run test sample plaque diameter to yield adjusted test plaque diameters for test-to-test comparisons. Repeatable results occurred when we excluded tests in which prototype control plaques were very crowded, had a high variance in diameter measurement, were unusually small, or numbered less than 25.

Plaquing efficiency. The $\log_{10}$ (SMicLD$_{50}$/Vero PFU) ratio is the plaquing efficiency index, which increases with relative inability of a virus sample to form plaques.

Virus passage and harvesting. We inoculated each chipmunk intraperitoneally and s. c. with 0.1 ml field mosquito suspension by each route. Table 1 shows virus doses and numbers of chipmunks inoculated by each strain. Daily whole blood samples taken from the retroorbital sinus were frozen in portions at −60 to −100 °C. Blood, generally from the day of maximum viremia titer, was thawed and fed to *Ae. triseriatus* mosquitoes through a membrane. After 28—39 days extrinsic incubation, we extracted saliva (Aitken, 1977) from surviving mosquitoes. Medium 199 with 10% heat-inactivated fetal calf serum was used as a saliva diluent and for subsequent trituration of mosquitoes with glass beads. Saliva and mosquito suspensions were frozen; individual virus-positive samples, as shown by i. c. suckling mouse inoculation, were pooled, divided into portions, and refrozen for later testing. No other passages were made in chipmunks or in mosquitoes.

Results

Neurovirulence studies

Mouse neurovirulence of four LAC virus field strains (77—88, 76—22, 76—26, 76—40) varied substantially during passage through natural hosts (Table 2). The indices for these strains changed by at least 1.0 at some time during passage. The indices of the prototype virus changed by 0.8, a more moderate variation. No substantial change occurred for two fields strains (77—92, 77—151); however, the limited neurovirulence studies of these two strains included neither the whole field mosquito suspensions nor the laboratory mosquito salivas.

Low mosquito saliva virus titers allowed only estimates of minimum possible neurovirulence indices for field strains 77—88 and 76—40. For strains 76—22 and 76—26, however, saliva virus titrations included endpoints by both s. c. and i. c. routes. For these two strains, neurovirulence indices of mosquito salivary virus were moderately but consistently higher than those of mosquito head and thorax virus, indicating lower neurovirulence.

Table 2. Neurovirulence indices of LaCrosse virus field isolates sequentially passed into chipmunks and then into colonized Aedes triseriatus mosquitoes

Strain	Field mosquito	Passage				
		Chipmunk blood		(N)	Laboratory mosquito Head and torso	Saliva
77—88	0.8		1.6	(1)	2.1	≧1.4
77—92	NT		1.6	(1)	1.3	NT
77—151	NT		1.5	(12)	1.1	NT
76—22	NT	A	2.2	(6)	1.1	2.0
		B	≧2.4	(1)	1.3	2.0
76—26	NT	A	0.7	(7)	1.4	≧2.2
		B	0.9	(3)	≧1.4	2.3
76—40	0.6	A	0.6	(7)	2.4	≧0.9
		B	1.4	(6)	1.4	1.6
Prototype	0.8*		1.6	(1)	1.6	0.8

Neurovirulence index: $\log_{10}$ (i.c./s.c.) titer in 8—9 day old mice.
(N): Number of pooled infected colonized *Ae. triseriatus* mosquitoes.
NT: Not tested.
A,B: Two different chipmunks infected by same virus strain.
* Prototype strain used initially after 6 passages in mice and 1 passage in Vero cells.

Changes in neurovirulence of other strains were inconsistent, with no clear pattern of selection following passage. When a single field strain was passed into two chipmunks and thence to their respective groups of feeding mosquitoes, within-strain neurovirulence changes were similar for the two parallel passage lines for two strains (77—22 and 76—26). However, strain 76—40 neurovirulence appeared to change in different patterns as it infected two different chipmunks and sets of mosquitoes. It remained high following passage into chipmunk A, and then declined markedly upon passage into laboratory mosquitoes. However, when strain 76—40 was inoculated into chipmunk B, its virulence declined, and then remained unchanged on subsequent passage into laboratory mosquitoes.

Plaque size studies

Passage in natural hosts consistently changed plaque diameter within each field strain, but with no overall consistent between-strain pattern (Table 3). By analysis of variance and subsequent t-test of the field strains, changes between each passage level were significant ($P < 0.05$) for strain 77—124, and highly significant ($P < 0.05$) for all the rest. For most field strains, chipmunk virus produced smaller plaques than did either field or laboratory mosquitoes. Strain 76—26 was exceptional; both chipmunks infected with this strain produced larger plaques than did their respective lots of feeding mosquitoes. Strain 76—40 yielded inconsistent changes when passed once through each of two different chipmunks and then into laboratory mosquitoes. In one experiment, the mean plaque size of this strain

Table 3. Plaque diameters of LaCrosse virus field isolates sequentially passed into chipmunks and then into colonized Aedes triseriatus mosquitoes

Strain	Field mosquito $\bar{x} \pm$ S.D. (N)	Chipmunk blood $\bar{x} \pm$ S.D. (N)	Passage No.	Laboratory mosquito Head and torso $\bar{x} \pm$ S.D. (N)	Laboratory mosquito Saliva $\bar{x} \pm$ S.D. (N)
77—88	2.34 ± 0.57 (43)	0.91 ± 0.41 (12)	1	3.87 ± 1.46 (148)	NT
77—92	NT	0.86 ± 0.46 (51)	1	4.11 ± 1.44 (106)	NT
77—124	1.49 ± 0.84 (7)	0.87 ± 0.20 (11)	0	NT	NT
77—151	NT	3.34 ± 0.75 (27)	12	4.93 ± 1.32 (47)	NT
76—22	1.62 ± 0.58 (10)	1.02 ± 0.32 (40)	6	3.65 ± 0.85 (28)	3.65 ± 0.86 (6)
76—26	NT	A 1.89 ± 0.37 (11)	7	1.07 ± 0.78 (42)	NT
		B 3.75 ± 0.61 (13)	3	1.33 ± 0.50 (8)	NT
76—40	3.88 ± 1.10 (21)	A 2.11 ± 0.63 (78)	7	0.89 ± 0.91 (21)	NT
		B 2.15 ± 0.82 (12)	6	4.67 ± 1.01 (100)	NT
Prototype	1.14 ± 0.76 (387)*	1.20 ± 0.22 (25)	1	1.04 ± 0.29 (2.52)	0.82 ± 0.15 (5)

$\bar{x} \pm$ S.D. (N): Mean adjusted plaque diameter ± 1 standard deviation (number of plaques measured)
No.: Number of pooled infected colonized *Ae. triseriatus* mosquitoes
NT: Not tested
A,B: Two different chipmunks infected by same virus strain
* Prototype strain used initially after 6 passages in mice and one passage in Vero cells

became successiveiy smaller following passage in chipmunk A and then into mosquitoes. The mean plaque size of the same strain 76—40 also became smaller following chipmunk B passage, but then more than doubled in size after subsequent mosquito passage.

In contrast, no significant change in prototype LAC virus plaque size occurred with passage ($P > 0.05$). For both the prototype and the 76—22 strains, the only ones for which mosquito saliva plaques were measured, salivary virus was not demonstrably different from that of the rest of the mosquito.

Plaquing efficiency studies

For all strains, chipmunk blood virus was consistently less efficient at forming plaques than either mosquito passage (Table 4). In 7 of 12 tests with 8 LAC virus isolates, $SMicLD_{50}$ titers of viremic chipmunk blood were over 100-fold greater than Vero cell PFU titers for chipmunk blood samples.

The relative difficulty of forming plaques from infected chipmunk blood might not be a function of variant selection, but instead a function of non-specific plaque inhibitors in chipmunk blood. To test this hypothesis prototype LAC virus grown in tissue culture was mixed with uninfected chipmunk whole blood, serum, or washed cells diluted in medium 199 with 10% fetal calf serum. Blood was pooled from ten animals. Plaque titers were reduced most by whole blood diluted 1 : 10, but not more than threefold. Blood or

Table 4. Plaquing efficiency of LaCrosse virus field isolates sequentially passed into chipmunks and then into colonized Aedes triseriatus mosquitoes

Strain	Field mosquito	Chipmunk blood	(N)	Passage Laboratory mosquito Head and torso	Saliva
77–88	0.2	1.4	(1)	0.4	≧0.7
77–92	NT	≧2.5	(1)	1.3	NT
77–124	0.2	1.2	(0)	NT	NT
77–151	NT	≧2.0	(12)	1.8	NT
76–22	1.5	A_1 2.3	(4)	0.9	NT
		A_2 2.5	(6)	1.7	2.2
		B 1.9	(0)	NT	NT
76–26	NT	A 2.1	(7)	1.3	1.7
		B 1.8	(3)	1.4	≧0.8
76–40	0.5	A 2.3	(7)	0.5	NT
		B 2.2	(6)	1.1	1.2
Prototype	0.6*	1.4	(1)	1.0	0.9

Plaquing efficiency: $\log_{10}$ (SMicLD$_{50}$/Vero PFU), adjusted to equal volumes.
(N): Number of pooled infected mosquitoes.
NT: Not treated.
A,B: Two different chipmunks infected by same virus strain.
A_1,A_2: Same chipmunk bled on 2 consecutive days.
* Prototype strain used initially after 6 passages in mice and 1 in Vero cells.

blood fractions diluted 1 : 100, a dilution comparable to actual experimental conditions, reduced plaque titers up to twofold. By the same test, the ratio of large to small plaque numbers was unchanged.

Discussion

These studies show that passage through natural hosts changes previously unpassaged LAC virus field isolates. We assume that these changes reflect selection of varying virus subpopulations. Passage of LAC virus into chipmunks consistently reduced plaquing efficiency for all strains. This consistent change agrees with the observations of Issel *et al.* (1975) on Jamestown Canyon virus passaged through its natural vertebrate host, the white-tailed deer (*Odocoileus virginianus*). Plaque size also tended to be significantly smaller at the chipmunk passage level than at either mosquito passage, although two field strains and the prototype virus were inconsistent with this trend. Mouse neurovirulence changed markedly but unpredictably with passage in natural hosts.

The underlying mechanisms causing these changes are unclear. The changes do not appear to have been due to inherent test variability or non-specific inhibitors. Titrations were consistently repeatable within 0.5 log units for both PFU and SMicLD$_{50}$ assays. Replicated neurovirulence indices for five different specimens differed by no more than 0.1 for four of them, and 0.6 for the fifth. By t-test, differences in replicated plaque size assays of six different specimens were insignificant ($P > 0.1$) and of marginal

significance for a single one ($0.02 < P < 0.05$). Uninfected chipmunk blood and blood fractions do not non-specifically reduce plaquing efficiency or the ratio of large to small plaques to any important extent.

By ruling out inherent test error and non-specific blood effects, we conclude that passage in natural hosts selects for LAC virus subpopulations. However, the unpredictability of neurovirulence and plaque size change suggests a complex interaction of selecting factors, one of which is host species. Repeated laboratory passage may remove some of this complexity so that change is more predictable or absent. For instance, the prototype LAC virus had undergone several serial passages in suckling mouse brain, a procedure known to select for small plaque size. In contrast with all seven of our LAC field strains, the plaque size of this prototype strain did not change during passage in chipmunks and *Ae. triseriatus*. Hansen (1976) reached the same conclusion using the same mouse-passaged prototype LAC virus for serial transmission to chipmunks and *Ae. triseriatus*. In both studies, prior mouse passage had not removed plaque variability; 10%—20% of plaques were large. However, mouse passage had apparently removed subpopulations of plaque size variants which could be selected for by natural hosts.

The biologic significance of LAC virus change in natural hosts is that change implies a perpetual variability in natural virus populations. This variability between strains has been noted by Ksiazek and Yuill (1977*a*). In general, the maintenance of variability and ability to change is important for the evolutionary survival of any type of organisms, presumably including arboviruses.

The diagnostic and laboratory research significance of LAC virus change centers on plaquing efficiency. In epidemiologic field studies, cell cultures could conceivably be used as an inexpensive system for virus isolation from mosquito suspensions. But suckling mice should be used as by far the more sensitive system to detect LAC virus in vertebrate blood. Laboratory studies must take into account the fact that frequently fewer than one in a hundred virus particles in viremic blood causes a detectable Vero cell plaque. For instance, plaque-picking is sometimes not feasible because of the strong possibility of contamination by non-plaquing virus particles; studies such as ours depend on the avoidance of the laboratory passages which would be required for plaque purification.

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References

Aitken, T. H. G. (1977): In vitro feeding technique for artificially demonstrating virus transmission by mosquitoes. *Mosq. News* **37**, 130—133.

Gauld, L. W., Hanson, R. P., Thompson, W. H., and Sinha, S. K. (1974): Observations on a natural cycle of LaCrosse virus in Southwestern Wisconsin. *Amer. J. trop. Med. Hyg.* **23**, 983—992.

Gauld, L. W., Yuill, T. M., Hanson, R. P., and Sinha, S. K. (1975): Isolation of LaCrosse virus (California Encephalitis group) from the chipmunk *Tamias striatus*, an amplifier host. *Amer. J. trop. Med Hyg.* **24**, 999—1005.

Hansen, W. R. (1976): The role of wildlife hosts in the selection of LaCrosse virus variants. Ph. D. thesis, Dept. of Veterinary Science, University of Wisconsin-Madison, Madison, Wisconsin, U.S.A.

Issel, C. J., Pantuwatana, S., Yuill, T. M., and Hanson, R. P. (1975): Selection for plaque variants of two California group arboviruses (Jamestown Canyon and LaCrosse) by passage in natural vertebrate hosts. *Acta virol.* **19**, 318—326.

Ksiazek, T. G., and Yuill, T. M. (1977*a*): Selection of LaCrosse virus variants by sentinel squirrels *(Sciurus carolinensis)* and chipmunks (*Tamias striatus*). *Acta virol.* **21**, 119—127.

Ksiazek, T. G., and Yuill, T. M. (1977*b*): Viremia and antibody response to LaCrosse virus in sentinel gray squirrels (*Sciurus carolinensis*) and chipmunks (*Tamias striatus*). *Amer. J. trop. Med. Hyg.* **26**, 815—821.

Málková, D. (1971): Virulence of Ťahyňa virus in mice and its relation to thermosensitivity and character of plaque population markers. *Acta virol.* **15**, 473—478.

Málková, D. (1974): Comparative study of two variants of Ťahyňa virus. *Acta virol.* **18**, 407—415.

Málková, D., and Reddy, G. N. (1975): Influence of early passages on the character of freshly isolated strains of Ťahyňa virus. *Acta virol.* **19**, 333—339.

Moulton, D. W., and Thompson, W. H. (1971): California group virus infections in small, forest-dwelling mammals of Wisconsin: Some ecological considerations. *Amer. J. trop. Med. Hyg.* **20**, 474—482.

Pantuwatana, S., Thompson, W. H., Watts, D. M., and Hanson, R. P. (1972): Experimental infection of chipmunks and squirrels with LaCrosse and trivittatus virus and biological transmission of LaCrosse virus by *Aedes triseriatus* mosquitoes. *Amer. J. trop. Med. Hyg.* **21**, 476—481.

Sukhavachana, P., Yuill, T. M., and Russell, P. K. (1969): Assay of arbovirus neutralizing antibody in micromethods. *Trans. roy. Soc. trop. Med. Hyg.* **63**, 446—455.