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Molecular basis of bunyavirus *per os* infection of mosquitoes: Role of the middle-sized RNA segment

(bunyavirus recombinant/gene function/midgut infection)

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ABSTRACT **The molecular basis of bunyavirus *per os* infection of mosquitoes was determined; La Crosse (LaC), snowshoe hare (Ssh), and LaC–Ssh reassortment viruses were compared for their ability to infect *Aedes triseriatus*, the natural vector of the LaC virus. The viruses were comparable in their ability to infect midgut cells; 115 of 117 (98%) mosquitoes ingesting viruses containing the LaC middle-sized RNA segment and 92/100 (92%) of mosquitoes ingesting viruses containing the Ssh middle-sized RNA segment became infected. However, those viruses containing the LaC middle-sized RNA segment disseminated efficiently (113/115, 98%) from the midgut to infect secondary target organs. Those viruses containing the Ssh middle-sized RNA segment efficiently infected the midgut and large amounts of viral antigen were detected in the midgut cells but antigen was detected in the secondary target organs only in 26% (24/92) of the mosquitoes with midgut infection. Thus, the middle-sized RNA segment seems to be the major determinant for successful dissemination of LaC virus from infected *A. triseriatus* midgut cells.**

Mosquito-borne arboviruses are typically restricted in their range of vector species (1, 2). The most fundamental and initial arbovirus–vector interaction involves the midgut. On ingestion of a viremic blood meal, the virus must infect and replicate in midgut epithilial cells (1, 2). Progeny virus must then disseminate from the midgut cells into the hemocoele. Only then can the epidemiologically important secondary target organs such as salivary glands and ovaries become infected (1–3). The subject of this report is the delineation of the bunyavirus gene(s), which functions to permit efficient vector midgut infection and subsequent dissemination of infection to secondary target organs.

Arboviruses of the family Bunyaviridae provide a unique opportunity to examine virus gene contributions to arbovirus–vector interactions. The bunyavirus genome is tripartite, containing large, middle-sized, and small RNA segments (4). Certain bunyaviruses of the California group are capable of segment reassortment *in vitro*. These reassortant viruses were used to define bunyavirus gene-coding assignments. The bunyavirus middle-sized RNA segment was found to code for the glycoproteins, the small RNA codes for the nucleocapsid, and the large RNA presumably codes for the polymerase (4). Reoviruses also contain segmented genomes, which has permitted a genetic approach to define the viral molecular basis for *in vivo* virulence and tissue tropism (5–7). We report here a similar approach to determine the bunyavirus molecular basis of *per os* infection of mosquito vectors.

One bunyavirus, La Crosse (LaC) virus, is a major cause of arthropod-borne encephalitis in the United States (8). The mosquito *Aedes triseriatus* is the vector. Snowshoe hare (Ssh) virus is serologically closely related to LaC virus but it has a distinct natural cycle, involving different *Aedes* species and *Culiseta inornata* vector mosquitoes and a different vertebrate host (9). Nonetheless, the two viruses are capable of segment reassortment both *in vitro* (4) and *in vivo* (10). The LaC, Ssh, and LaC–Ssh reassortant viruses were subsequently used to determine the molecular basis of bunyavirus transmission by mosquitoes (11). In this study, we report the use of the LaC–Ssh parent and reassortant viruses to determine the molecular basis for bunyavirus infection of mosquitoes.

METHODS AND MATERIALS

Mosquitoes. *A. triseriatus* mosquitoes (F_3–F_5 generation) were obtained from a colony maintained at the Yale Arbovirus Research Unit. This colony originated from eggs collected in Wisconsin by Wayne Thompson of the University of Wisconsin. Mosquitoes were maintained at 22°C, 60% relative humidity, and a 16-hr:8-hr photoperiod. Sucrose (10%) was provided ad lib. Mosquitoes were sucrose deprived for 24 hr before the infection attempts.

Viruses. The parent and reassortant viruses used are shown in Table 1. Viruses are described according to their large/middle-sized/small segment composition. Thus, a virus designated LaC/LaC/Ssh derived its large and middle-sized segments from the LaC parent and its small segment from the Ssh parent. The origin of the plaque-cloned LaC and Ssh viruses has been described (4).

Immunofluorescence The immunofluorescence technique was used to detect viral antigen in mosquito tissues (3, 12). To prepare the conjugate, high-titer anti-LaC antibodies were prepared by hyperimmunization of mice. Ascitic fluids were collected by paracentesis, and antibodies were precipitated with $(NH_4)SO_4$ and conjugated with fluorescein isothiocyanate (12). The conjugate was then titrated and proven capable of detecting both parental viral antigens in mosquito tissues and organ dissections.

Preparation of Infectious Blood Meals. Blood meals were typically composed of equal parts of washed human erythrocytes, a 10% sucrose/fetal calf serum mixture, and the respective virus suspension. Meal suspensions were thoroughly mixed, incubated at 37°C for 30 min, and presented to the mosquitoes in cotton pledgets. A portion of each meal was held at room temperature for the duration of the feeding attempt. This virus was stored at −70°C and subsequently analyzed in a microtitration test using BHK-21 cells.

Experimental Design. Approximately 50 mosquitoes were exposed to each infectious blood meal. After 14 days of extrinsic

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Abbreviations: LaC, La Crosse; Ssh, snowshoe hare.

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Table 1. Infection of *A. triseriatus* mosquitoes by LaC and Ssh parent and reassortant viruses

Virus		Midgut infection†		Dissemination to secondary organs‡	
Genome	Titer*	No./no.	%	No./no.	%
LaC/LaC/LaC	6.8	44/45	98	44/44	100
LaC/LaC/Ssh	6.8	25/27	93	3/25	12
Ssh/LaC/LaC	6.8	32/32	100	31/32	97
Ssh/LaC/Ssh	7.1	39/40	98	38/39	97
Total		140/144	97	116/140	83
Total excluding LaC/LaC/Ssh§		115/117	98	113/115	98
Ssh/Ssh/LaC	6.3	38/40	95	11/38	29
LaC/Ssh/LaC	6.8	19/21	90	8/19	42
LaC/Ssh/Ssh	6.8	12/13	92	1/12	8
Ssh/Ssh/Ssh	7.3	23/26	89	4/23	17
Total		92/100	92	24/92	26

* Log $TCID_{50}$ per ml.
† Results represent positive (no. having detectable viral antigen in midgut or abdominal squash)/examined (no. surviving 14 days of extrinsic incubation).
‡ Results represent positive (no. having detectable viral antigen in head squash or dissected secondary organs)/negative (no. having detectable antigen in midgut or abdominal squash).
§ LaC/LaC/Ssh excluded because of putative silent mutation(s) in one or more segment(s).

incubation, a portion of each group of mosquitoes was cold anesthetized, after which the heads were severed and squashed on slides. In addition, major organ systems of the abdomen (midgut, dorsal vessel, abdominal ganglia) were dissected and examined by immunofluorescence techniques to determine the anatomic localization of the virus (3, 12). Heads and abdomens of the nondissected mosquitoes were severed and squashed on slides. Detection of viral antigen in head tissues indicated that the mosquito had become infected and also that the infection had disseminated from the midgut tissues and infected secondary target organ systems. Detection of viral antigen in abdominal tissues only indicated that viral antigen was probably restricted to the midgut tissues and that virus had not disseminated to infect secondary target organs.

RESULTS

Infection of Midgut Cells. All parent and reassortant viruses were capable of infection of *A. triseriatus* midgut cells (Table 1). Both the midgut dissections and the abdominal squash preparations showed large amounts of viral antigen in the infected mosquitoes. There was no statistical difference in the ability of the viruses to infect the midgut cells ($\chi^2 = 3.44$; $P > 0.05$). Infection rates ranged from 100% (32/32) for Ssh/LaC/LaC to 89% (23/26) for Ssh/Ssh/Ssh. The mean midgut infection rate for all viruses was 95% (232/244).

Dissemination from Midgut Cells. In contrast to midgut infection, there was a marked difference in the ability of the viruses to escape from the midgut cells or to infect secondary target organs (or both) (Table 1). With the exception of LaC/LaC/Ssh virus, viruses containing the LaC middle-sized RNA segment (113/115, 98%) were significantly more efficient ($\chi^2 = 118.96$; $P < 0.001$) in establishing a disseminated infection than viruses containing the Ssh middle-sized RNA segment (24/92, 26%). Although midgut infection by Ssh middle-sized RNA segment viruses was substantial, as evidenced by the accumulation of viral antigen in midgut cells, antigen was detected in the secondary target organs of only 26% of the infected mosquitoes. The organ dissections showed that viral antigen was indeed restricted to midgut cells; the dorsal vessel, abdominal ganglia, and ovaries were all in general concordance with detection of antigen in the head squash preparation. However, when the mosquito was infected with a virus containing a LaC middle-sized RNA segment that resulted in a disseminated infection, the majority of organ systems contained large amounts of viral antigen.

There also seemed to be a difference in the ability of the viruses containing the Ssh middle-sized RNA segment to disseminate from the midgut. Those viruses containing the LaC small RNA segment (19/57, 33%) were somewhat more efficient ($\chi^2 = 6.62$; $P < 0.05$) in the establishment of disseminated infections than the viruses containing the Ssh small RNA segment (5/35, 14%).

LaC/LaC/Ssh was markedly different (for reasons discussed below) from the other viruses containing the LaC middle-sized RNA segment in its ability to establish a disseminated infection. Only 3 of 25 (12%) of the infected mosquitoes contained detectable viral antigen in secondary organ systems.

DISCUSSION

A genetic approach using wild-type and reassortant viruses of known RNA segment composition was used to explore the molecular basis of bunyavirus infection of mosquitoes. The middle-sized RNA segment that codes for the viral envelope glycoproteins is associated with more efficient dissemination of virus from infected midgut cells to secondary target organs, a critical step in arbovirus–vector interactions.

The apparent equivalent capability of the parent and reassortant viruses to infect *A. triseriatus* midgut cells was surprising. Evidently, if efficient infection of midgut cells is principally mediated by specific receptors, then the receptor is not sufficiently discriminatory to distinguish between the LaC and the Ssh middle-sized RNA gene products—the glycoproteins. However, it must be noted that the virus titers used in these studies were considerably higher than those found in the blood of viremic hosts (13). Such large quantities of virus may have overwhelmed natural thresholds of infection and thus obscured receptor differences that would be apparent at more biologically relevant titers. Dose–response studies need to be conducted to determine whether differential infection does occur at lower virus titers.

In contrast, those viruses containing the LaC middle-sized RNA were more efficient in establishing a disseminated infection. In mosquitoes infected with Ssh virus, substantial amounts of viral antigen were detected in the midgut and abdominal

squash preparations. Since dissected secondary target organs and head tissues infrequently contained antigen, apparently the virus replicated in midgut cells but did not efficiently disseminate to infect secondary target organs. A similar phenomenon was previously described in which Ssh middle-sized RNA viruses were less efficiently orally transmitted than LaC middle-sized RNA viruses by parenterally infected mosquitoes (11). This occurred even when Ssh middle-sized RNA virus antigen was detectable in the salivary glands. Thus, it seems reasonable to speculate that Ssh middle-sized RNA gene products either inefficiently interact with *A. triseriatus* cell membranes or that the glycoproteins are poorly recognized by the small RNA gene product—the nucleocapsid protein—of either parent virus. Alternatively, there may be differences in the rate of replication of the two types of viruses in mosquito cells. Increased extrinsic incubation periods may result in increased dissemination rates for the Ssh middle-sized RNA segment viruses.

The altered ability of the LaC/LaC/Ssh virus to disseminate from infected *A. triseriatus* midgut cells is suspected to be due to the presence of a silent mutation(s) in one or more of the RNA segments. The reassortant virus was derived by using 5-fluorouracil mutagenesis and has been shown to have originally contained a silent mutation in the large RNA segment (14). The virus has reduced pathogenicity for mice (14) and is less efficiently transmitted by *A. triseriatus* mosquitoes (11).

The role of the small RNA segment in dissemination of viruses containing the middle-sized RNA segment remains to be clarified. Viruses containing the LaC small RNA were marginally more efficient in dissemination from the midgut than those containing the Ssh small RNA. Perhaps the LaC small RNA does enhance the ability of a virus containing the Ssh middle-sized RNA to disseminate. However, there remains the possibility that the LaC/Ssh/Ssh virus, which was markedly less efficient in dissemination than the other viruses (Table 1), may also contain a silent mutation(s).

The applicability of these findings to the maintenance of the integrity of arbovirus cycles in nature remains to be determined. It should be determined whether the rates of infection and dissemination are reversed when a natural vector of Ssh virus is used. Such results would establish the role of the middle-sized RNA segment as a major determinant of bunyavirus–vector interactions.

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