POTENTIAL FOR EVOLUTION OF CALIFORNIA SEROGROUP BUNYAVIRUSES BY GENOME REASSORTMENT IN Aedes albopictus

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Abstract. Aedes albopictus was introduced into the United States in used tires in 1985. Its successful colonization of the upper Midwest has potential to alter the current epidemiology of bunyaviruses that circulate in the region. It is permissive for the replication of several arboviruses, including La Crosse (LACV) and Jamestown Canyon (JCV) bunyaviruses. In this study, we demonstrate the ability of LACV and JCV to coinfect Ae. albopictus mosquitoes and to form all six possible reassortant genotypes. All reassortant viruses infect Ae. albopictus orally and can be transmitted to suckling mice. All reassortants are neurovirulent in mice. However, reassortant viruses carrying the LACV M segment in the foreign genetic background of JCV are more neuroinvasive than JCV, or any other reassortant genotype. In addition, these reassortants can replicate in gerbils and infect Ae. triseriatus, characteristics of LACV, but not JCV. Because Ae. albopictus is spreading into new geographic areas and feeds on a variety of mammals, including humans, it has the potential to transmit new, emerging bunyaviruses in nature.

The recent introduction of Aedes albopictus, the Asian tiger mosquito, into the Western hemisphere is of concern because it is a competent vector of several arboviruses of human and animal health significance.1,2 Moreover, this species could play a role in the emergence of new, reassortant bunyaviruses in areas such as the midwestern United States. Aedes albopictus has been spreading into regions where Jamestown Canyon virus (JCV) and La Crosse virus (LACV) are highly endemic in wild mammal populations.3 Both LACV and JCV, members of the family Bunyaviridae, can replicate in Ae. albopictus.4 This mosquito species is a particularly aggressive pest that commonly feeds on humans, as well as a wide variety of mammalian species, including the natural hosts for LACV and JCV.5

These two viruses are of public health significance. La Crosse virus is the cause of most pediatric cases of arboviral encephalitis in the United States.6 Jamestown Canyon virus has recently been recognized as a cause of human meningitis and encephalitis, primarily in adults.7,8 Like other California serogroup viruses, LACV (California encephalitis complex) and JCV (Melao subgroup) circulate in specific transmission cycles consisting of a restricted number of vertebrate hosts and a single or a few preferred mosquito vectors. Aedes triseriatus, a woodland mosquito, is the principal vector for LACV and may transmit the virus by bite and transovarially or venereally.9,10 In contrast, JCV is transmitted by several species of Aedes, but not by Ae. triseriatus.11 The amplifying hosts for LACV are squirrels and chipmunks, whereas the primary vertebrate hosts for JCV are white-tailed deer.11-13 These two viruses are often sympatric in nature, but the integrity of their specific transmission cycles is remarkably retained.

Because the bunyavirus genome is segmented, it can reassort during a mixed infection. The bunyavirus genome consists of three single-stranded segments of negative-sense RNA. Each segment is associated with nucleocapsid protein, and enclosed by a host-derived lipid envelope that contains the two virion glycoproteins (G1 and G2). The three RNA segments are the large segment (L), which codes for the polymerase protein; the middle segment (M), which codes for the two virion glycoproteins, G1 and G2, and a nonstructural protein; and the small segment (S), which codes the nucleocapsid protein (N) and a second nonstructural protein.14-19 There has not been any report of reassortment between LACV and JCV. Failure to find reassortant viruses in nature may be due to the fact that LACV and JCV are maintained in separate transmission cycles involving different vertebrate hosts and vector mosquitoes, so that mixed infections are unlikely. Also, it is possible that the molecular differences in the genomes of these two viruses do not permit reassortment to occur.

Aedes albopictus has the potential to acquire mixed infections of bunyaviruses in nature. We hypothesized that dual infections of LACV and JCV in Ae. albopictus can occur and will generate reassortant viruses. We also proposed that the vertebrate host or mosquito vector specificity will be altered depending on the genomic composition of reassortants. Upon generation of reassortants in the laboratory, we compared their neurovirulence and neuroinvasiveness in mice, ability to produce viremia in a vertebrate host, infection efficiency of mosquitoes, and transmission from mosquitoes to mice. We present data to support our hypotheses and to demonstrate that reassortment in nature between LACV and JCV in Ae. albopictus may result in new bunyaviruses with altered pathogenicity as well as amplifying host range and mosquito specificity.

MATERIALS AND METHODS

Viruses and cells. Two California group viruses, LACV and JCV, were used in these studies. The LACV-77 strain was originally isolated from Ae. triseriatus mosquitoes in Iowa County, Wisconsin in 1977. Jamestown Canyon virus, obtained from the American Type Culture Collection (Rockville, MD) (ATCC #VR712), was isolated from Culiseta inornata captured at Greeley, Colorado. Vero cells (African green monkey kidney cells; ATCC #CCL-81) were grown in Eagle’s medium 199 (M199; Sigma Chemical Co., St. Louis, MO) supplemented with 5% fetal bovine serum (FBS) and maintained at 37°C in 5% CO2. The C6/36 cell line (ATCC #CRL-1660), originally derived from Ae. albopictus embryonic tissue, was grown in Leibovitz L-15 media supple-
mented with 10% tryptose phosphate broth and 5% FBS, and was maintained in closed flasks at 28°C. The C2C12 cell line (ATCC #CRL-1772), a murine muscle cell line, was used as an indicator for myotropism and neuroinvasiveness, and was maintained as previously described. Working stocks of each virus were prepared on monolayers of Vero cells. A multiplicity of infection (MOI) of 0.01 was used to infect cells. Culture supernatant was removed from infected cell cultures when a 90% cytopathic effect (CPE) was observed, and clarified by centrifugation. Viruses used in the experiments were propagated in Vero cells no more than four passages.

Probe preparation. Recombinant plasmids containing complementary DNA (cDNA) copies of the LACV L (pLAC 4.16), M (pLAC 4.27), or S (pLAC 4C-26) RNA segments were used to prepare hybridization probes in these experiments. Plasmid DNA was purified from Escherichia coli cells according to standard procedures. For pLAC 4.16 and pLAC 4.27, the cDNA inserts were digested with the restriction endonuclease Bam HI; for pLAC 4C-26, the plasmid was digested with Pst I. The cDNA inserts were separated by electrophoresis in 0.8% agarose gels, recovered by filtration through cellulose acetate membrane disposable filters, and then labeled with digoxigenin by random-primed DNA synthesis (Genius® System; Boehringer Mannheim, Indianapolis, IN). For the preparation of the probe for JCV, because there were only partial sequences of JCV M segment available when this study was conducted, a 41-basepair oligonucleotide probe specific for the JCV M segments was synthesized at the Biotechnology Center, University of Wisconsin-Madison. The nucleotide sequence 5'-TATGGAAGTCCTAAACTTAATCTGGTTACTAGCATTGTGCT-3' is based on the sequence of the JCV M RNA (provided by Dr. C. Huang, State Health Department, Albany, NY). The oligonucleotide was 3'-end labeled with digoxigenin using a Genius® System kit (Boehringer Mannheim).

Northern hybridization to test LACV cDNA probe specificity was performed with total RNA preparations from LACV, JCV, or mock infected Vero cells. Briefly, 95% monolayers of Vero cells were inoculated with LACV or JCV at an MOI of 30–50 and incubated for 3–4 hr at 37°C. Total RNA was extracted by the standard guanidine thiocyanate method, precipitated with ethanol, and resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Total RNA (8 μg/lane) was electrophoresed for 3–4 hr in a 0.8% agarose gel in 1× MOPS (0.2 M MOPS, 0.5 M sodium acetate, 0.01 M EDTA) and 6% (v/v) formaldehyde, transferred to nylon membranes, and then fixed at 80°C for 2 hr. Blots were treated in Church buffer for 2–3 hr at 42°C in a hybridization oven (Hybaid; National Labnet Co., Woodbridge, NJ) and subsequently hybridized in Church buffer with individual digoxigenin-labeled probes for 16–18 hr at 42°C. Blots were washed under stringent conditions: twice in 2× SSC (0.3 M NaCl, 30 mM trisodium citrate), 0.1% sodium dodecyl sulfate (SDS) at room temperature and twice in 0.1× SSC, 0.1% SDS at 48°C (LACV L and M probes, and JCV M oligonucleotide) or 65°C (LACV S probe). Following washing, blots were incubated in maleate buffer (0.1 M maleic acid, 0.15 M NaCl; pH 7.5) with 0.3% Tween 20 for 5 min, and incubated in 1% (w/v) blocking buffer (Boehringer Mannheim) for 1 hr. After blocking, blots were in-cubated in the same blocking buffer with alkaline phosphatase-conjugated anti-digoxigenin (diluted 1:10,000) (Boehringer Mannheim) for 90 min at room temperature. After two 15-min washes in maleate buffer with 0.3% Tween 20, the membranes were transferred to Genius 3 buffer (0.1 M Tris-HCl, 0.1 M NaCl, 50 mM MgCl₂, pH 9.5) and then immersed in Lumigen PPD® (C₄₀H₃₂O₁₆PNa₁₁) (Boehringer Mannheim) diluted in Genius 3 buffer. Blots were exposed to x-ray film for up to 3 hr the following day.

Generation of reassortants and cytoplasmic RNA extraction. La Crosse virus and Jamestown Canyon virus were coinoculated onto monolayers of C6/36 cells at an MOI of 2–5 or cofed to mosquitoes (see Mosquito Studies section) to generate reassortant viruses. Supernatant was removed from infected cell cultures after incubation at 28°C for 72 hr and was clarified by centrifugation at 1,200 rpm for 20 min. Plaque assays were performed in Vero cells. After four days, various sizes of plaques were selected for an additional round of plaque purification.

Virus suspensions prepared from individual plaques were inoculated onto single wells of a six-well tissue culture plate containing a 95% confluent monolayer of Vero cells. Infected cells were incubated until 50–60% CPE was observed (approximately 48 hr). Cytoplasmic RNA was extracted from the cells as previously described. Samples were stored at −80°C until the time of analysis.

Slot blot hybridization. To analyze the genotype of each progeny virus isolate, the RNA samples were blotted onto four sets of MagnaGraph nylon membrane (Micron Separations, Inc., Westborough, MA) and hybridized individually with LACV cDNA probes specific for L, M, or S RNA segments or a JCV oligo probe for M segment.

Hybridization was performed under the same stringent conditions as Northern hybridization. The genotype of progeny viruses was determined by the hybridization signal with LACV cDNA probes on the film. Because these probes do not hybridize to JCV at stringent conditions, the presence of signal indicated the segment was from LACV parental virus, while the absence of signal indicated the segment was obtained from a JCV parental virus.

Radioimmunoprecipitation (RIP) assay. The genotype of reassortant viruses was confirmed by examining the viral protein migration on SDS-polyacrylamide gels. Viral lysates were labeled with 35S-methionine and 35S-cysteine, and used in the RIP assay as previously described. Following precipitation, viral proteins were separated by electrophoresis on 8.75% discontinuous SDS-polyacrylamide gels at 40 mA/gel for 3–4 hr. Proteins were transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA) using a semidy transfer apparatus at 156 mA for 135 min. Membranes were stained with amido black to view the position of molecular weight markers and then exposed to film (X-omat; Eastman Kodak, Rochester, NY) for 2–4 days.

Virus neutralization assay. The parental origin of the M segment of reassortant viruses was also confirmed by neutralization assay. Two monoclonal antibodies (MAbs) against G1 glycoprotein of LACV (8C2.2) and JCV (M3; neutralizing MAb provided by Dr. Harvey Arthos, Laboratory Centre for Disease Control, Ottawa, Ontario, Canada) was serially diluted 1:2 in M199 in 96-well microtiter plates (Costar/Corning, Acton, MA). Stock virus was diluted in M199, and
100 50% tissue culture infectious doses of diluted virus were added to triplicate wells at each dilution of MAF. Virus and MAF were incubated at 37°C for 1 hr, and then 2 × 10^4 Vero cells in 100 μl of M199, 5% FBS, and 2× antibiotics (200 units/ml of penicillin, 0.2 mg/ml of streptomycin, and 0.5 μg/ml of amphotericin B) were added to each well. The controls consisted of a cell control without MAF or virus, a back titration of the virus, and irrelevant MAF as a negative control. The plates were incubated for four days and scored microscopically for the presence or absence of CPE. The MAb neutralization titer was defined as the last dilution where no more than one of the three wells was positive for CPE.

**Gerbil infectivity study.** Because of the difficulties of obtaining and handling the natural wild vertebrate amplifying hosts in the laboratory, we used Mongolian gerbils (*Meriones unguiculatus*) as a laboratory vertebrate host model for susceptibility to LACV and JCV. Previous studies and our preliminary results have shown that gerbils are susceptible to LACV infection, survive infection, and develop viremias comparable to those seen in adult chipmunks. Four- to six-week-old gerbils were inoculated intramuscularly (IM) with a mixture of LACV and JCV in a 37°C water-jacketed membrane feeder fitted with fresh mouse skin. Only fully engorged mosquitoes were kept after the feeding. After a 14-day extrinsic incubation period, mosquitoes were collected and processed individually for the recovery of reassortants. To determine the efficiency of oral infectivity and transmission of viruses, each mosquito species was fed with LACV, JCV, or reassortants as described above. Several mosquitoes from each group were processed individually and inoculated into cell cultures for virus isolation to determine the oral infectivity efficiency of the viruses. The remaining mosquitoes were used in the transmission study. Mosquitoes were placed in cages (5–10 per cage) and those in each cage were allowed to feed on a two- to three-day-old suckling mouse. After feeding, suspensions of individual mosquitoes were inoculated into cell cultures to confirm infection with virus. The mice were observed for 15 days for illness or death as evidence of virus transmission, and the infection of mice was confirmed by detection of viral antigen by IFA in mouse brain smears.

**Replication of viruses in C2C12 cells.** The C2C12 myocyte cultures were grown in Dulbecco’s modified Eagle medium (DMEM) in 15% FBS. When the mononuclear cells grew into nearly confluent monolayers, the medium was changed to DMEM with 2% FBS for 5–7 days to induce myogenic differentiation. At this time, more than 50% of the C2C12 myocyte cultures became myotubes. Myotubes cultures were infected with LACV, JCV, and reassortants generated from C6/36 cells at an MOI of 1. Infected cells were examined 24-hr postinoculation for viral antigen using IFA staining with LACV G2 MAb (3D9.4).

**Neuroinvasiveness studies.** Suckling mice (five mice per dose) were inoculated subcutaneously with LACV, JCV, or all six reassortant viruses (generated from *Ae. albopictus*) at doses of 1, 10, 50, and 100 PFU. The mice were observed three times per day for illness or death for a 15-day period. Mouse brain smears were fixed as previously described and tested by IFA to confirm the presence or absence of viral antigen. The method of Reed and Muench was used to calculate the 50% lethal dose (LD_{50}) endpoint titers of neuroinvasiveness in mice.

**Mosquito studies.** After JCV and LACV were shown to reassort in vitro in C6/36 cells, experiments were conducted to determine if reassortment would occur in the potential vector mosquito, *Ae. albopictus*, as well. The New Orleans strain of *Ae. albopictus* and the Walton strain of *Ae. triseriatus* (originally collected as larvae from La Crosse, WI) were provided by Dr. B. Beaty (Colorado State University, Fort Collins, CO). Mosquitoes were reared in a walk-in environmental chamber at 26.5 ± 0.5°C and 80 ± 5% relative humidity on a 16 hr light : 8 hr dark cycle. Adults were maintained on 0.3 M sucrose-impregnated cotton. To generate reassortant viruses, we fed mosquitoes on specific antibody-free defibrinated rabbit blood containing 5.0–7.0 logs^{10/ml} of a mixture of LACV and JCV in a 37°C water-jacketed membrane feeder fitted with fresh mouse skin. Only fully engorged mosquitoes were kept after the feeding. After a 14-day extrinsic incubation period, mosquitoes were collected and processed individually for the recovery of reassortants. To determine the efficiency of oral infectivity and transmission of viruses, each mosquito species was fed with LACV, JCV, or reassortants as described above. Several mosquitoes from each group were processed individually and inoculated into cell cultures for virus isolation to determine the oral infectivity efficiency of the viruses. The remaining mosquitoes were used in the transmission study. Mosquitoes were placed in cages (5–10 per cage) and those in each cage were allowed to feed on a two- to three-day-old suckling mouse. After feeding, suspensions of individual mosquitoes were inoculated into cell cultures to confirm infection with virus. The mice were observed for 15 days for illness or death as evidence of virus transmission, and the infection of mice was confirmed by detection of viral antigen by IFA in mouse brain smears.

**RESULTS**

**Generation and identification of reassortants of LACV and JCV in vitro and in vivo.** Four of six possible genotypes of reassortants between LACV and JCV were isolated from C6/36 cells. The genotypes, designated by the parental origin of L/M/S RNA segments were JCV/LACV/JCV, LACV/LACV/JCV, LACV/JCV/JCV, and JCV/JCV/LACV. A total of 938 plaques from four dually infected C6/36 preparations were analyzed. A representative autoradiograph for four reassortant viruses as well as the parental virus and cell controls is shown in Figure 1. The specificity of each probe
was confirmed by Northern hybridization prior to their use in the studies. The distribution of the reassortant genotypes from C6/36 cells is summarized in Table 1.

All six possible genotypes of reassortants between LACV and JCV were found in dually infected Ae. albopictus. A total of 294 plaques were analyzed. The distribution of the reassortant genotypes is summarized in Table 1.

**Confirmation of reassortant genotype by immunoprecipitation of viral proteins and virus neutralization assay.** Protein migration on SDS-polyacrylamide gels confirmed the identity of reassortant viruses generated from dually infected C6/36 cells. Polyvalent MAF against JCV was used to immunoprecipitate lysates from JCV-, LACV-, or reassortant-infected cells (Figure 2). Due to cross-reactivity of viral proteins, MAF against JCV bound the G1 glycoprotein (encoded by the M segment) and nucleocapsid protein (encoded by the S segment) of LACV as well as of JCV. However, the differences in the relative mobility of these proteins on gels allowed us to differentiate LACV from JCV. The four representative reassortants from C6/36 cells used in the RIP assay also illustrate a pattern of viral protein migration that reflects which viral RNA segments each virus contains (Figure 2, lanes 4–7). Additionally, virus neutralization assays were conducted to confirm the parental origin of the M segment (Table 2). The LACV G1 MAb (8C2.2) effectively neutralized LACV but not JCV. In contrast, JCV was effectively neutralized by MAb M3 and LACV was not. Because the virion glycoproteins encoded by the M segment are responsible for the neutralization of virus, when anti-LACV G1 MAb was used, reassortants carrying LACV M segment were effectively neutralized, whereas reassortants with an M segment from JCV were neutralized by MAb M3.

**Duration of viremias in gerbils.** Gerbils inoculated with LACV or reassortants with LACV M segment became viremic after a longer period than those infected with JCV, consistent with our previous observations (Table 2).

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**Table 1**

Distributions of reassortant viruses of La Crosse virus (LACV) and Jamestown Canyon virus (JCV) resulting from dual infection of C6/36 cells (derived from *Aedes albopictus*) and *Ae. albopictus* mosquitoes

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>No. of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Segment)</td>
<td>C6/36 cells</td>
</tr>
<tr>
<td>L</td>
<td>L</td>
</tr>
<tr>
<td>L</td>
<td>M</td>
</tr>
<tr>
<td>L</td>
<td>S</td>
</tr>
<tr>
<td>J</td>
<td>L</td>
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<td>J</td>
<td>L</td>
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<td>J</td>
<td>L</td>
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<tr>
<td>L</td>
<td>L</td>
</tr>
<tr>
<td>J</td>
<td>L</td>
</tr>
<tr>
<td>Total isolates</td>
<td>938</td>
</tr>
</tbody>
</table>

**Table 2**

Monoclonal antibody (MAb) neutralization of La Crosse virus (LACV), Jamestown Canyon virus (JCV), and reassortant viruses generated in C6/36 cells

<table>
<thead>
<tr>
<th>Viruses (L/M/S RNA segments)</th>
<th>Anti-LACV G1 MAb*</th>
<th>Anti-JCV G1 MAb†</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAC/LAC/LAC</td>
<td>&gt;1:6,400‡</td>
<td>&lt;1:50</td>
</tr>
<tr>
<td>J/C/J/C</td>
<td>&lt;1:50</td>
<td>&gt;1:6,400</td>
</tr>
<tr>
<td>LAC/LAC/JC</td>
<td>&gt;1:6,400</td>
<td>&lt;1:50</td>
</tr>
<tr>
<td>J/C/LAC/JC</td>
<td>&gt;1:6,400</td>
<td>&lt;1:50</td>
</tr>
<tr>
<td>LAC/J/C</td>
<td>&lt;1:50</td>
<td>&gt;1:6,400</td>
</tr>
<tr>
<td>J/C/J/LAC</td>
<td>&lt;1:50</td>
<td>&gt;1:6,400</td>
</tr>
</tbody>
</table>

* Designated 8C2.2.
† Designated M3.
‡ The highest dilution of MAb that completely neutralizes 100% tissue culture infectious virus dose.
Table 3
Duration of viremias in gerbils inoculated with La Crosse virus (LACV), Jamestown Canyon virus (JCV), and four reassortants* assayed in suckling mice

<table>
<thead>
<tr>
<th>Gerbil inoculum</th>
<th>Gerbil serum†</th>
<th>Day 0</th>
<th>Day 2</th>
<th>Day 4</th>
<th>Day 6</th>
<th>Day 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock</td>
<td>NT</td>
<td>0/6</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>LAC/LAC/LAC</td>
<td>0/12</td>
<td>4/12</td>
<td>12/12</td>
<td>2/12</td>
<td>0/12</td>
<td>0/12</td>
</tr>
<tr>
<td>JC/JC/JC</td>
<td>0/12</td>
<td>0/12</td>
<td>0/12</td>
<td>0/12</td>
<td>0/12</td>
<td>0/12</td>
</tr>
<tr>
<td>LAC/LAC/JC</td>
<td>0/12</td>
<td>0/12</td>
<td>2/12</td>
<td>6/12</td>
<td>0/12</td>
<td>0/12</td>
</tr>
<tr>
<td>JC/LAC/JC</td>
<td>0/6</td>
<td>1/6</td>
<td>5/6</td>
<td>1/6</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td>LAC/JC/JC</td>
<td>0/6</td>
<td>0/12</td>
<td>0/12</td>
<td>0/12</td>
<td>0/12</td>
<td>0/12</td>
</tr>
<tr>
<td>JC/JC/LAC</td>
<td>0/6</td>
<td>0/12</td>
<td>0/12</td>
<td>0/12</td>
<td>0/12</td>
<td>0/12</td>
</tr>
</tbody>
</table>

* Reassortants used in the studies were generated from C6/36 cells.
† Serum was collected from gerbils on days 0, 2, 4, 6, and 8 following intramuscular inoculation with virus. Values are the no. of dead mice/total no. of mice inoculated intracerebrally with 10^3 ml of gerbil serum. NT = not tested.

Remic. Jamestown Canyon Virus and reassortants carrying the M segment from JCV did not cause detectable viremia in gerbils. We used the four reassortants generated from C6/36 cells in the gerbil studies. Because the two additional reassortant genotypes had not been isolated when these experiments were conducted, these two reassortants were not tested in gerbils (Table 3). Mice inoculated with sera from LACV-inoculated gerbils (days 2–6 postinoculation) died, but mice inoculated with sera from JCV-injected gerbils survived. The presence or absence of viral antigen in the brains of dead or killed sucking mice was confirmed by IFA using anti-G1 MAb to either LACV (8C2.2) or JCV (M3). Viral antigens were detected only in the brains of mice that had been inoculated with sera from gerbils injected with either LACV or a reassortant virus containing the LACV M segment. During the course of these experiments, gerbils did not show overt signs of central nervous system disease. To ensure our murine indicator system was permissive for all viruses tested, we also inoculated parental and reassortant viruses IC into suckling mice. All viruses were neurovirulent for suckling mice, which died within three days after the injection, and viral antigen was present in the brain tissue as determined by indirect immunofluorescence assay. All parental and reassortant viruses caused mortality following IC inoculation with 1–10 PFU of the virus.

Control of neuroinvasiveness by the M segment. We tested the differentiated murine muscle cell line C2C12 as an indicator for neuroinvasiveness as reported by Griot and others, and found that there was a difference in the replication of viruses carrying the M segment of LACV or JCV (Figure 3). As previously described, differentiated myocytes expressed myosin as shown by immunofluorescent staining for myosin heavy chains while most mononuclear myocytes in the same cultures did not.

Replication in C2C12 cells correlates positively with neuroinvasiveness in suckling mice. All six reassortants used in the neuroinvasiveness studies were generated from Ae. albopicus. Our results indicate that LACV is more neuroinvasive than JCV and that reassortant viruses with the M segment of LACV origin have a neuroinvasiveness profile similar to that of LACV. We designated the reassortants with LACV M segments as group I viruses and reassortant viruses carrying JCV M segments as group II viruses. In Figure 4, the data patterns clearly show differences in mouse neurovirulence between viruses with LACV M segments and those with JCV M segment. When the JCV M segment is present, a higher dose of viruses is required to kill mice, and time until death is prolonged. Data from group I and group II viruses were significantly different (P < 0.001) when analyzed by two-way analysis of variance (viral groups versus

Figure 3. Indirect immunofluorescent staining of cultures 24 hr after infection at a multiplicity of infection of 1 showing that viruses with an M RNA segment derived from La Crosse virus (LACV) infected a higher proportion of C2C12 murine myotubes than did viruses carrying a Jamestown Canyon virus (JCV)–derived M segment. Reassortants used in the study were generated from C6/36 cells.
FIGURE 4. Neuroinvasiveness studies in suckling mice (five mice/group) inoculated subcutaneously with 1, 5, 10, or 100 plaque-forming units (PFU) of La Crosse (LAC) and Jamestown Canyon (JC) viruses, and reassortants generated in Aedes albopictus mosquitoes. LD$_{50}$ = 50% lethal dose; P.I. = postinfection.

Group I Viruses -- LACV M segment

Group II Viruses -- JCV M segment

viral doses). In addition, the LD$_{50}$ of viruses with LACV M segment ranged from 2 to 4 PFU, whereas the LD$_{50}$ of viruses carrying JCV M segment ranged from 8 to 70 PFU (Figure 4).

Control of mosquito infection and transmission by the M segment. The ability of reassortants to infect mosquitoes orally was related to the origin of the M segment. All six reassortants used in the studies were generated from Ae. albopictus. La Crosse virus and reassortants containing LACV M segment infected Ae. triseriatus mosquitoes, the natural vector, whereas JCV and reassortants carrying JCV M segment did not. Parental LACV and JCV, and all reassortants
infected *Ae. albopictus*, although JCV and reassortants with JCV M segment infected *Ae. albopictus* at approximately half the rate that was observed with LACV and reassortants containing LACV M segment (Table 4). In addition, infected mosquitoes transmitted parental as well as reassortant viruses to suckling mice (Table 4). The transmission of virus was confirmed by detection of viral antigen in mouse brains as previously described.

**DISCUSSION**

The spread of *Ae. albopictus* to new geographic areas and ecosystems could play an important role in the emergence of viral diseases. *Aedes albopictus* has been shown experimentally to be a competent vector for a number of arboviruses that are of considerable medical or veterinary importance, including dengue, Japanese encephalitis, yellow fever, and several California encephalitis group bunyaviruses.2-4

The segmented nature of the genomes of bunyaviruses can give rise to new viruses through reassortment of gene segments in dually infected individuals. Studies have demonstrated the potential for superinfection in mosquitoes when the period between initial and subsequent infective blood meals is less than 48 hr.30 In addition, feeding by some mosquito species in nature is easily interrupted by host defensive behavior, which can result in an individual mosquito taking blood meals from more than one viremic host.31 The opportunity for dual bunyavirus infection to occur in *Ae. albopictus* is enhanced by its aggressive nature as an opportunistic feeder on a variety of mammals, including humans. Thus, the establishment of *Ae. albopictus* in areas where bunyaviruses occur sympatrically possesses the possibility that new, reassorted viruses could be generated that may be of human, domestic animal, or wildlife health significance.

Our studies have demonstrated that reassortants between LACV and JCV can be generated both *in vitro* in an *Ae. albopictus* cell line (C6/36), as well as *in vivo* in *Ae. albopictus*. Of more biological significance, all reassortants can *in vitro* in both mosquito and mammalian cells, and *in vivo* in mosquitoes that are dually infected.24,30,32,33 In addition, analysis of field isolates suggests that reassortment also can occur in nature.34-36 However, most of these reassortants had been limited to closely related bunyaviruses within a subgroup of the same serogroup, except one report with limited laboratory generated reassortant genotypes between viruses from California encephalitis (CE) subgroup and trivittatus (TVT).37,38 Our work provides further evidence that more distantly related bunyaviruses can also reassort. In this study, four of six possible reassortant genotypes were generated *in vitro*, and all six genotypes were generated during replication in mosquitoes. The reassortment was not random, with a predominance of parental viruses and the genotypes JCV/LACV/JCV (*in vitro*) and JCV/LACV/LACV, JCV/LACV/JCV, and LACV/LACV/JCV (*in vivo*). At this time, we do not know why the preponderant reassortants from the *in vitro* system are different from those recovered from the *in vivo* system. Evidence for nonrandom reassortment between the tripartite RNA bunyavirus genomes and restricted reassortment has been reported previously.32,33 The predominance of reassortants containing homologous L and M segments and M and S segments, as previously reported among LACV and snowshoe hare (SSH) virus or Tahyna (TAH) virus,23,33,39 did not occur in our system. However, because LACV and JCV represent different antigenic subgroups, in contrast to the higher sequence identity between LACV and SSH viruses or TAH viruses,20,41 the occurrence of homologous segment associations might be less likely.

Although it is difficult to predict what all the characteristics of new reassortant viruses might be, the origin of the M segment is likely to determine which vector and host species the virus will be able to infect and how severe the resulting disease may be. We observed that gerbils infected with viruses containing the LACV M segment became viremic, whereas those containing the JCV M segment did not. For example, replacement of the JCV M segment by the LACV M segment results in the genotype JCV/LACV/LACV, which was dominant among the reassortant viruses generated in C6/36 cells. This reassortant virus infected gerbils as well as *Ae. triseriatus* in contrast to the JC parental virus, which could not infect either. The reassortants LACV/JCV/JCV and JCV/JCV/LACV were unable to infect gerbils or *Ae. triseriatus*, despite the fact that one segment was of LACV origin and that LACV infects this vertebrate host and mosquito species easily. It would be interesting to know if reassortants with JCV M segment would produce a viremia in white-tailed deer adequate to infect mosquito vectors efficiently.

Similarly, neurovirulence and neuroinvasiveness were related to the LACV M segment, irrespective of the origin of the L and S segments. We have shown that viruses carrying the LACV-derived M segment can replicate more rapidly in C2C12 murine myotubes cultures compared with viruses bearing the M segment from JCV. This difference correlates strikingly to the neuroinvasiveness profile in sucking mice. Reassortants carrying the LACV M segment kill sucking mice faster and with a lower inoculation dose than do reassortants with JCV M segment. Earlier studies by others, using cell culture–generated reassortants between LAC and TAH viruses, have shown that the M RNA segment of LACV is the major determinant of virulence in mice, with the other two segments influencing the phenotype.42,43

Our study has at least three significant findings: first,
LACV and JCV can reassort in *Ae. albopictus* despite their being in different antigenic subgroups, which implies a wide range of potential reassortant combinations of California serogroup bunyaviruses in nature; second, reassortment of LACV and JCV can alter the vertebrate amplifying host specificity, and expand the mosquito vector species for the viruses; and third, *Ae. albopictus* could serve as a potential site for the evolution of California group viruses by genome segment reassortment. *Aedes albopictus* is recognized for its broad viral susceptibility, its rapid colonization of artificial aquatic larval habitats, and tolerance of diverse physical conditions.\(^2,3\) Since its initial introduction, *Ae. albopictus* has spread to more than 25 states and into populated areas of the United States where LACV and JCV are sympatric. The spread to more than 25 states and into populated areas of \[^{4}\]yaviruses or their reassortants merit close surveillance.

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