A Recombinant Chimeric La Crosse Virus Expressing the Surface Glycoproteins of Jamestown Canyon Virus Is Immunogenic and Protective against Challenge with either Parental Virus in Mice or Monkeys

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La Crosse virus (LACV) and Jamestown Canyon virus (JCV), family *Bunyaviridae*, are mosquito-borne viruses that are endemic in North America and recognized as etiologic agents of encephalitis in humans. Both viruses belong to the California encephalitis virus serogroup, which causes 70 to 100 cases of encephalitis a year. As a first step in creating live attenuated viral vaccine candidates for this serogroup, we have generated a recombinant LACV expressing the attachment/fusion glycoproteins of JCV. The JCV/LACV chimeric virus contains full-length S and L segments derived from LACV. For the M segment, the open reading frame (ORF) of LACV is replaced with that derived from JCV and is flanked by the untranslated regions of LACV. The resulting chimeric virus retained the same robust growth kinetics in tissue culture as observed for either parent virus, and the virus remains highly infectious and immunogenic in mice. Although both LACV and JCV are highly neurovirulent in 21 day-old mice, with 50% lethal dose (LD_{50}) values of 0.1 and 0.5 $\log_{10}$ PFU, respectively, chimeric JCV/LACV is highly attenuated and does not cause disease even after intracerebral inoculation of 10^3 PFU. Parenteral vaccination of mice with 10^1 or 10^3 PFU of JCV/LACV protected against lethal challenge with LACV, JCV, and Tahyna virus (TAHV). The chimeric virus was infectious and immunogenic in rhesus monkeys and induced neutralizing antibodies to JCV, LACV, and TAHV. When vaccinated monkeys were challenged with JCV, they were protected against the development of viremia. Generation of highly attenuated yet immunogenic chimeric bunyaviruses could be an efficient general method for development of vaccines effective against these pathogenic viruses.

La Crosse virus (LACV), which belongs to the family *Bunyaviridae*, is a pathogenic member of the California encephalitis virus (CEV) serogroup in the genus *Orthobunyavirus*. The serogroup contains members found on five continents that include human pathogens such as LACV, Snowshoe hare virus, and Jamestown Canyon virus (JCV) in North America, Guarao virus in North and South America, Inkoo virus and Tahyna virus (TAHV) in Europe, and Lumbo virus in Africa (22). LACV is transmitted by hardwood forest dwelling mosquitoes, *Aedes triseriatus*, which breed in tree holes and outdoor containers. *A. triseriatus* feed on Eastern chipmunks (*Tamias striatus griseus*) and Eastern gray squirrels (*Sciurus carolinensis*) which serve as amplifying hosts for LACV (1, 11, 21). More recently, LACV has been isolated from naturally infected, nonnative *Aedes albopictus* mosquitoes (13). The infection of *A. albopictus* may represent a shift in virus ecology with the potential to expand the area of endemicity and increase the possibility for generation of new reassortants in nature (9).

The CEV serogroup results in 70 to 130 recognized cases of central nervous system (CNS) disease a year in the United States and is the leading cause of pediatric arboviral encephalitis (8, 17, 19, 27). In children and adults, severe LACV encephalitis clinically mimics herpes simplex virus encephalitis with fever, focal signs, and progression to coma in some instances (18, 20, 29). Confirmatory diagnosis has been made by RT-PCR of cerebrospinal fluid to exclude herpes simplex virus and by fluorescent staining for LACV antigen in brain biopsy sections (20). Children who recover from severe LACV encephalitis may have significantly lower IQ scores than expected and a high prevalence (60% of those tested) of attention deficit-hyperactivity disorder (19). Seizure

disorders are also common in survivors (2). Projected lifelong economic costs associated with neurologic sequelae range from \$48,775 to \$3,090,000 per case (30).

Members of the CEV serogroup, including LACV, contain a genome consisting of three single-stranded, negative-sense RNA genome segments designated small (S), medium (M), and large (L). The S segment encodes two proteins in overlapping reading frames: the nucleoprotein (N) and a nonstructural protein (NS_S) that suppresses type 1 interferon (IFN) in the mammal host. The M segment encodes a single polypeptide (M polypeptide) that is posttranslationally processed into two glycoproteins (G_N and G_C), and a nonstructural protein of unknown function (NS_M) (23, 24). G_N and G_C are the major proteins that elicit neutralizing antibodies (12, 28). The L segment encodes a single open reading frame (ORF) for the RNA-dependent RNA polymerase (L) (26).

LACV is genetically related to JCV, which is endemic to the United States, TAHV found throughout Europe and Asia, and a variant called Lumbo virus in Africa (22). In the United States, it is not clear how many California serogroup virus infections can be attributed to JCV infection, but some estimates suggest that up to

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62% of presumed LACV diagnoses are actually JCV (15). Like its North American counterparts, TAHV can also result in CNS infection; however, no fatalities have been reported (16). Currently, a vaccine or specific antiviral treatment for the CEV serogroup is not available, but one could serve to reduce the clinical and economic impact of these common infections.

To be both economically feasible and efficacious, a vaccine would need to offer protection against multiple members of the CEV serogroup. We have previously reported that infection of rhesus monkeys with JCV results in a strong cross-neutralizing antibody response effective against JCV, LACV, and TAHV that was not observed following infection with LACV or TAHV (3). As a first step in vaccine development, we sought to generate a recombinant LACV virus (rLACV) that expresses the G_N and G_C glycoproteins of JCV, which should induce neutralizing antibodies against JCV, LACV, and TAHV. Here, we report the early development of a live attenuated orthobunyavirus vaccine candidate that is highly attenuated for the CNS of mice yet efficacious in both mice and monkeys. In addition, the use of the LACV genetic background to deliver the G_N and G_C protective antigens from pathogenic bunyaviruses other than JCV could be an efficient method for the development of vaccines effective against other pathogenic bunyaviruses that require biosafety level 3 (BSL3) or BSL4 containment.

MATERIALS AND METHODS

Cells and viruses. Vero (African green monkey kidney) cells were maintained in OptiPRO SFM medium (Invitrogen) supplemented with 4 mM L-glutamine. C6/36 cells (*Aedes albopictus* mosquito larvae) were maintained in Earle's minimal essential medium (MEM) (Invitrogen) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 2 mM L-glutamine (Invitrogen), and 1 mM nonessential amino acids (Invitrogen). BHK-21 clone BSR T7/5 cells which express T7 polymerase under neomycin resistance (7) were grown in MEM supplemented with 2 mM L-glutamine, 2% MEM amino acid solution (Invitrogen), and 10% fetal bovine serum (FBS). Cells were selected for T7 expression by growth in the presence of Geneticin at a concentration of 1 mg/ml of medium every other passage.

JCV used in this study was a biological clone of the prototype strain 61V2235 (JCV/61/CO-cl) and previously sequenced and characterized (4). The recombinant LACV (rLACV) was generated from cDNA sequence of LACV strain 78 V 13193 (LACV/78/NC-cl) and was previously sequenced and characterized (5).

Genetic construction of rLACV and recombinant JCV/LACV chimera (rJCV/LACV). LACV/78/NC-M, a mosquito isolate of LACV previously demonstrated to be fully virulent in mice and infectious for rhesus monkeys, was chosen as a genetic background for the development of a reverse genetics system. The complete genome was divided into five overlapping fragments, one on the S segment, two on the M segment, and three on the L segment. Viral RNA isolation, reverse transcription, and PCR using high-fidelity Platinum PFX (Invitrogen) were used to generate cDNA fragments for subcloning into five separate plasmids that were each sequenced to verify that they matched the parental virus genome. A silent change, G4081A, was left in the L segment to allow for differentiation of wild-type and recombinant viruses (see Table S1 in the supplemental material). The subcloned fragments were reconstructed into full S, M, and L segments on separate plasmids with each genome segment flanked by a T7 polymerase promoter sequence and the hepatitis delta virus ribozyme as previously described (6, 10). To generate an rLACV virus expressing the glycoproteins and NS_M of JCV virus, the M ORF of LACV was replaced with that of JCV/61/CO-cl using standard molecular cloning techniques. The resulting M segment of the rJCV/LACV chimera contains the 3' and

5' untranslated region (UTR) of LACV with the ORF encoding the G_N , NS_M , and G_C proteins of JCV (see Fig. S1 in the supplemental material).

Both rLACV and rJCV/LACV were recovered in BHK cells that express T7 polymerase. Briefly, 2 μ g of each of the three plasmids containing the S, M, and L genome segments were transfected into BHK monolayers (50 to 70% confluent) using TransIT-LT1 (Mirus) and incubated at 32°C or 35°C for up to 5 days. Recombinant viruses were terminally diluted three times and amplified one additional time in Vero cells as previously described (5). Virus stocks were fully sequenced as described below.

Sequence analysis of viral genomes. Viral RNA was isolated using a MasterPure RNA purification kit (Epicentre Biotechnologies, Madison, WI). Overlapping PCR fragments were generated using a Titan One Tube RT-PCR kit (Roche) using JCV- or LACV-specific primers. PCR fragments were purified and both strands were directly sequenced using virus-specific primers in BigDye terminator cycle sequencing reactions analyzed with an ABI3730 genetic analyzer (Applied Biosystems, Foster City, CA). Sequence fragments were assembled into a consensus sequence using AutoAssembler 2.1 software (Applied Biosystems).

Virus titration. Serial dilutions of tissue culture fluid or serum were added to confluent monolayers of Vero cells and incubated for 1 h to allow for virus attachment. Cells were overlaid with 1% methylcellulose in Opti-MEM (Invitrogen) and incubated for 5 days at 37°C. After incubation, the overlay was removed, and the monolayers were washed twice with phosphate-buffered saline (PBS) and stained with crystal violet to allow for the enumeration of virus plaques.

In vitro growth kinetics. Confluent monolayers of C6/36 or Vero cells were infected at a multiplicity of infection (MOI) of 0.01 and incubated for 1 h to allow virus to attach to the cells. Infected monolayers were washed thrice with sterile PBS and overlaid with medium. Vero cell cultures were incubated at 37°C for 4 days. Tissue culture fluid (0.45 ml) was collected every 24 h after infection, mixed with a one-tenth volume of 10 $\times$ SPG buffer (final concentration, 218 mM sucrose, 6 mM L-glutamic acid, 3.8 mM dibasic potassium phosphate, pH 7.2), and flash frozen on dry ice. Samples were titrated as described above.

Virus inoculation of mice. The 50% lethal dose (LD_{50}) of JCV, rLACV, and rJCV/LACV viruses were evaluated using 21- to 23-day-old (weanling) outbred Swiss Webster mice (Taconic Farms, Germantown, NY). All animal experiments were carried out in accordance with the regulations and guidelines of the National Institutes of Health. Weanling mice ($n = 6$ mice/dose group) were inoculated with serial dilutions of biologically cloned virus in a volume of 10 μ l intracerebrally (i.c.) or 100 μ l intraperitoneally (i.p.). The mice were anesthetized with isoflurane prior to i.c. inoculation. All mice were carefully observed twice daily for clinical disease, including lethargy, circling, tremors, limb paralysis, and general moribundity. Mice exhibiting these signs of disease were promptly euthanized. Because clinically moribund mice were humanely euthanized before succumbing to infection, moribundity served as a surrogate for the determination of lethality. For calculations of the mouse 50% infectious dose (ID_{50}), mice were considered infected if they either developed clinical disease or demonstrated a 4-fold increase in antiviral neutralizing antibody titers (see below for the neutralization assay). To determine the level of protection elicited by vaccination, mice were immunized by i.p. inoculation with rJCV/LACV at 21 to 23 days of age as described above and challenged i.c. 28 days later (49 to 52 days old) with JCV, LACV, or TAHV.

Virus growth in the mouse CNS. Three-week-old weanling mice were inoculated with 100 PFU of JCV, rLACV, or rJCV/LACV, and the brain and spinal cord were collected for virus titration. Tissues were collected on days 1 to 4 for JCV and rLACV since mice did not survive after that time. Tissues from rJCV/LACV-infected mice were collected for 8 days postinoculation. Tissues were homogenized in L15 medium at a 1:10 (wt/vol) ratio for the brain and a 1:100 (wt/vol) ratio for the spinal cord. Limits of detection were 1.7 log₁₀ PFU/g for the brain and 2.7 log₁₀ PFU/g for the spinal cord.

To assess the genetic stability of the rJCV/LACV chimera, virus present

FIG 1 Growth kinetics in Vero cells. Growth kinetics of JCV, rLACV, and rJCV/LACV in Vero cells infected at an MOI of 0.01. Each point represents the mean titer for three replicates. The standard error of any mean is ≤ 0.28 .

in mouse brains harvested on days 5 and 6 was amplified once in Vero cells, and viral genomes were sequenced as described above.

rJCV/LACV efficacy against challenge with JCV, LACV, and TAHV in mice. Three-week-old weanling mice ($n = 18/\text{dose}$) were inoculated with 10^1 or 10^3 PFU rJCV/LACV or L15 medium intraperitoneally, and survival blood draws were collected from mice 26 days postinoculation to detect neutralizing antibody. Mice were challenged i.c. with lethal doses of JCV, LACV, and TAHV 28 days postinoculation with rJCV/LACV or L15 medium. All mice were carefully observed twice daily for clinical disease, including tremors and limb paralysis, and all moribund mice were humanely euthanized before they succumbed to infection. Mice were observed for 28 days postchallenge.

rJCV/LACV infectivity in monkeys. Twelve JCV- and LACV-seronegative rhesus monkeys were inoculated subcutaneously with 10^5 PFU of JCV ($n = 2$), rLACV ($n = 4$), rJCV/LACV ($n = 4$), or L15 medium ($n = 2$). All monkeys were observed daily for clinical signs of disease. Blood samples were collected on days -7 , 0 , 2 , 4 , 6 , 8 , 28 , and 42 postinoculation for determination of virus titer (viremia) or neutralizing antibody titer.

rJCV/LACV efficacy in monkeys against JCV challenge. Monkey previously immunized with JCV, rLACV, rJCV/LACV, or L15 medium were challenged subcutaneously with 10^5 PFU of JCV, and blood was collected on days 2 , 4 , 6 , 8 , 10 , or 28 postchallenge for determination of virus titer (viremia) or neutralizing antibody titer. All monkeys were observed daily for clinical signs of disease.

Neutralization assay. Neutralizing antibody in mouse and monkey serum was quantified by a plaque reduction neutralization titer (PRNT). Test sera were heat inactivated (56°C for 30 min), and serial 2-fold dilutions beginning at $1:10$ were prepared in Opti-MEM (Invitrogen) supplemented with 2% FBS, 50 $\mu\text{g}/\text{ml}$ gentamicin, and 0.5% human albumin (Talecris Biotherapeutics, Inc., Research Triangle Park, NC). JCV was diluted to a final titer of 500 PFU/ml in the same diluent containing 10% guinea pig complement (Cambrex Bioscience Walkersville, Inc.) and was added to equal volumes of the serum dilutions and mixed well. The serum/virus mixture was incubated at 37°C for 30 min, added to confluent monolayers of Vero cells that were incubated for 1 h to allow virus attachment. Cells were overlaid with 1% methylcellulose in Opti-MEM and incubated for 5 days at 37°C . After incubation, the overlay was removed, and the monolayers were washed twice with PBS and stained with crystal violet to allow for the enumeration of virus plaques. A 60% plaque-reduction neutralization titer was calculated.

RESULTS

Chimerization of JCV and LACV does not affect growth *in vitro*. Both rLACV and rJCV/LACV were recovered in BHK cells expressing T7 polymerase. Following transfection, recombinant vir-

us titers frequently reached 10^6 PFU/ml in 2 or 3 days (data not shown). Recombinant virus genomes were completely sequenced to identify the virus and to detect adventitious mutations that may have occurred during transfection and virus amplification (see Table S1 in the supplemental material). The L segment of both rLACV and rJCV/LACV contained the G4081A mutation that was introduced to differentiate the segment from wild-type LACV.

The rJCV/LACV virus grows rapidly in Vero cells infected at an MOI of 0.01. The rJCV/LACV maintains growth kinetics similar to those of the parental JCV and rLACV. Titers greater than 10^6 PFU/ml were obtained after 24 h, and peak titers greater than 10^7 PFU/ml were reached 48 h after infection (Fig. 1). There was no significant difference between virus titers on days 1 to 4 (Tukey-Kramer *post hoc* test, $P > 0.05$).

Chimeric rJCV/LACV is infectious and attenuated for mice.

Swiss Webster mice, 21 to 23 days old, were inoculated intracerebrally (i.c.) or intraperitoneally (i.p.) with serial dilutions of JCV, rLACV, or rJCV/LACV. Mice were observed for 21 days for the development of clinical disease, including lethargy, tremors, seizures, or paralysis. Since all mice exhibiting clinical disease were promptly euthanized, clinical disease requiring euthanasia served as a substitute for lethality. For ID_{50} calculations, mice were considered infected if they either developed clinical disease or if there was a 4-fold increase in neutralizing antibody titer. Wild-type JCV is highly infectious by either the i.c. or i.p. inoculation route but is not neuroinvasive after i.p. inoculation (Table 1). rLACV is both highly infectious and virulent after i.c. or i.p. inoculation, with as little as 10^{-1} PFU initiating a lethal infection (Table 1). rJCV/LACV was highly infectious but not virulent after i.c. or i.p. inoculation (Table 1). No clinical disease was observed with mice after rJCV/LACV inoculation. Thus, chimerization of the M segment of LACV with JCV resulted in an unanticipated reduction in neurovirulence but not in infectivity, properties of a virus that would make rJCV/LACV a good candidate for a live attenuated virus vaccine.

In the mouse CNS, rJCV/LACV replicates at lower levels than those of parental viruses. To determine if rJCV/LACV was indeed replicating in the mouse CNS, 21- to 23-day-old mice were inoculated i.c. with 10^2 PFU of JCV, rLACV, or rJCV/LACV, and the brain and spinal cord were harvested from six mice each day. Mice inoculated with JCV or rLACV survived for only 4 days after inoculation. Tissues were collected from mice inoculated with rJCV/LACV for 8 consecutive days. JCV and rLACV replicate to high titers in the mouse brain and spinal cord, reaching nearly 10^6

TABLE 1 Chimeric rJCV/LACV is attenuated and highly infectious in weanling Swiss Webster mice after i.c. or i.p. inoculation

Virus	Neurovirulence (i.c.) (log ₁₀ PFU) ^a		Neuroinvasiveness (i.p.) ^b	
	LD ₅₀	ID ₅₀ ^c	LD ₅₀	ID ₅₀ ^c
JCV	0.5	< -1.0	> 4	< 0
rLACV	0.1	0.1	1.6	1.6
rJCV/LACV-cl	> 3	< -1.0	> 4	0.2

^a Groups of six weanling mice, 21 to 23 days old, were inoculated intracerebrally with 10 μl of diluted virus (-1 to 3 log₁₀ PFU).

^b Groups of six weanling mice, 21 to 23 days old, were inoculated intraperitoneally with 100 μl of diluted virus (0 to 4 log₁₀ PFU).

^c Mice were considered infected with virus if they developed clinical disease or a 4-fold increase in neutralizing antibody titer.

FIG 2 Chimeric rJCV/LACV replicates at very low levels in the CNS of mice compared to those of parental JCV and rLACV. Mice were inoculated intracerebrally with 100 PFU of JCV, rLACV, or rJCV/LACV, and six mice were sampled from each treatment group each day. Data shown are the mean virus titers/g in the brain (A) or spinal cord (B) for each treatment group. All mice in the rLACV or JCV treatment groups had succumbed to disease prior to the day 5 sampling. The lower limit of virus detection was 1.7 log₁₀ PFU/g for brain samples and 2.7 log₁₀ PFU/g for spinal cord samples and is indicated by a dotted line. To assess the genetic stability of rJCV/LACV after replication in mouse brain, virus was isolated and sequenced on indicated days (↓).

PFU/g by day 4, and mice did not survive beyond day 4 for sampling (Fig. 2). rJCV/LACV replicated to levels which were barely detectable in the mouse CNS and were 1,000- to 10,000-fold lower than that of either parental virus. Thus, the attenuation of rJCV/LACV for the CNS of mice was due to a greatly reduced replication of virus.

rJCV/LACV is genetically stable after mouse brain passage.

To assess the genetic stability of the rJCV/LACV chimera, virus obtained from six mouse brains harvested on days 5 and 6 was amplified in Vero cells (Fig. 2). Three virus isolates had genome sequences identical to that of the inoculating virus (see Table S2 in the supplemental material). None of the isolates had mutations in the L segment. One isolate contained a single synonymous mutation in the S segment. One isolate contained a nonsynonymous change in both the S and M segments, and one isolate contained a synonymous change in both the S and M segments and an additional nonsynonymous change in the M segment. No changes in the 3' or 5' UTR of the M segment or the chimeric junctions were observed. Thus, four of the six isolates had no or only minimal genetic changes, whereas two isolates had changes that did not affect the attenuating chimeric regions of the virus, suggesting that the chimeric virus maintains its attenuation genetic alterations intact after 5 to 6 days of replication in the CNS.

Vaccination of mice with rJCV/LACV protects against JCV, LACV, and TAHV lethal challenge. Swiss Webster mice were inoculated i.p. with 10¹ or 10³ PFU of the rJCV/LACV chimera (*n* = 18/dose), and serum was collected 26 days later for neutralizing antibody titer against rJCV/LACV. Eighteen mice were mock inoculated with L15 medium, and serum was collected 26 days later to confirm that control mice remained seronegative for rJCV/LACV. Fifteen of 18 mice (83%) that received 10 PFU rJCV/LACV developed neutralizing antibody responses (Table 2). All 18 mice that were inoculated with 1,000 PFU of rJCV/LACV developed a neutralizing antibody response. Mice in groups of six were challenged on day 28 with a lethal i.c. dose of biologically cloned JCV, LACV, or TAHV and were observed for an additional 28 days for the development of clinical disease. The three mice that did not have a detectable neutralizing antibody response after primary rJCV/LACV inoculation succumbed to challenge (Table 2). Primary immunization with 10³ PFU of rJCV/LACV provided 100% protection against homologous and heterologous (LACV or TAHV) lethal challenge.

rJCV/LACV infection of rhesus monkeys is immunogenic and protective against JCV challenge. Rhesus monkeys were inoculated subcutaneously with 10⁵ PFU of JCV, rLACV, or rJCV/LACV, and blood was collected on days 2, 4, 6, 8, 10, 12, 28, and 42 postinoculation for detection of viremia. Inoculation of rhesus monkeys with rJCV/LACV resulted in a detectable viremia on days 2, 4, and 6 postinoculation but did not produce any clinical signs of disease (Table 3). The mean peak titer of viremia in rJCV/LACV-inoculated monkeys (0.8 ± 0.19 PFU/ml) was significantly lower (*t* test, *P* = 0.0032) than that observed with the parental JCV virus (2.1 ± 0.19 PFU/ml). As observed in previous studies, infection with rLACV did not result in detectable viremia. All monkeys developed a high level of neutralizing antibodies against the immunizing virus (Table 4). Inoculation with either JCV or rJCV/LACV elicited a cross-neutralizing antibody response that neutralized heterologous LACV and TAHV. However, immunization with LACV elicited detectable cross-neutralizing antibodies against TAHV, but not JCV.

Forty-two days postinfection, monkeys were challenged with JCV and blood was collected for detection of viremia. Monkeys were not challenged with LACV since this virus does not produce viremia or disease in monkeys, thus precluding an assessment of efficacy. Monkeys previously infected with JCV, rLACV, or rJCV/LACV

TABLE 2 Immunization with chimeric rJCV/LACV protects mice against i.c. challenge with JCV, LACV, or TAHV

Virus used for immunization ^a	Dose (PFU)	% seropositive ^b	No. of survivors after i.c. challenge with indicated virus/total no. of mice		
			JCV	LACV	TAHV
rJCV/LACV	10	83	5/6 ^c	6/6	4/6 ^c
rJCV/LACV	1,000	100	6/6	6/6	6/6
None (L-15)	0	0	0/6	0/6	0/6

^a Weanling Swiss Webster mice were inoculated intraperitoneally with 100 μl of rJCV/LACV or Leibovitz L-15 medium.

^b Mice with at least a 4-fold increase in vaccine-specific neutralizing antibodies (PRNT, >40) were considered seropositive.

^c Mortality occurred after challenge in mice that had a neutralizing antibody titer of <1:10, confirming that these mice had not been infected with rJCV/LACV at a dose of 1 log₁₀ PFU.

TABLE 3 Infection of rhesus monkeys with rJCV/LACV elicits sterilizing immunity against JCV challenge

Virus inoculum	Monkey no.	Viremia on indicated day (log ₁₀ PFU/ml) ^a			
		Primary infection ^b			Challenge ^c
		Day 2	Day 4	Day 6	Day 2
JCV	DBX2	2.4			
	DC3A	2.4		0.7	
rLACV	DBX5				
	DBTZ				
	DC27				
	DBTW				
rJCV/LACV	DBZ9	0.7			
	DBZN			0.7	
	DC98		1.0		
	DC15		0.7		
None (L-15)	EVO				1.6
	DBV1				2.0

^a Areas left blank indicate a virus titer below the limit of detection (0.7 log₁₀ PFU/ml).
^b Virus was not detected in any serum samples on days 8, 10, 28, or 42 post-primary infection.
^c Monkeys were challenged with 10⁵ PFU of JCV on day 42 postinoculation. Virus was not detected in any serum samples on days 4, 6, 8, 10, or 28 postchallenge.

LACV were protected against the development of JCV viremia (Table 3). Primary rLACV inoculation was able to prevent the development of JCV challenge viremia even in the absence of detectable JCV-neutralizing antibodies (Table 4). Although JCV challenge viremia was not detectable in any of the virus-immunized monkeys, it is evident by the 4-fold increase in JCV antibody titers following challenge of the LACV monkeys that most of these monkeys were infected with the challenge virus. Only a single monkey immunized with JCV or rJCV/LACV showed a 4-fold increase in JCV antibody titer following chal-

lenge, indicating that these monkeys were immune to JCV infection.

DISCUSSION

A successful live attenuated CEV serogroup vaccine would need to be safe and optimally protective against multiple virus species and replicate sufficiently well in tissue culture to allow for efficient manufacture. Our overall strategy to accomplish this goal was (i) to develop a reverse genetics systems to recover rLACV in a manner similar to that previously reported (6), (ii) to use LACV as a background for generating antigenic chimeric viruses in which the ORF of the LACV M segment was replaced with that of another CEV that causes human disease, and (iii) to incorporate attenuating mutations into the L or S segment of the rLACV if further attenuation is needed. As a first step in this process, we generated a recombinant chimeric JCV/LACV by replacing the protective G_N and G_C antigens of wild-type LACV with those of JCV by exchanging the LACV M ORF with that of JCV. It is known that generating antigenic chimeric flaviviruses between two virulent parental viruses can result in attenuation (25, 31), so we chose to initiate this project with wild-type JCV and LACV parental viruses to facilitate the detection of an attenuated rJCV/LACV since both parental viruses maintain measurable phenotypes (neuroinvasiveness and/or neurovirulence in mice). In this manner, the resulting level of attenuation can be readily determined by comparison to the nonattenuated parent viruses. JCV was chosen as donor of the M segment ORF for several reasons. First, an antibody response against JCV was shown previously to also efficiently neutralize LACV and TAHV (3); thus, a virus that contains the JCV glycoproteins may provide protection against a wider range of CEV serogroup viruses than the parental LACV. Second, JCV produces viremia in monkeys and is neurovirulent in mice, providing two animal models to evaluate the level of attenuation and efficacy of a recombinant chimeric JCV/LACV if it were obtained. Third, although JCV and LACV reassortants have not been identified in nature, previous research indicated that a reassortant iso-

TABLE 4 Vaccination of rhesus monkeys with chimeric rJCV/LACV elicits cross-neutralizing antibodies against LACV and TAHV

Virus inoculum	Monkey no.	Neutralizing antibody titer on indicated day against indicated virus (reciprocal PRNT ₆₀) ^a					
		Day 42 postinoculation			Day 28 postchallenge ^b (JCV)		
		LACV	TAHV	JCV	LACV	TAHV	JCV
JCV	DBX2	93	247	148	161	398	518
	DC3A	59	131	197	118	180	269
rLACV	DBX5	282	15	<10	555	1,061	156
	DBTZ	595	116	<10	866	2,217	24
	DC27	40	<10	<10	358	596	53
	DBTW	1,490	129	<10	976	2,020	152
rJCV/LACV	DBZ9	35	50	512	209	288	1,165
	DBZN	32	132	139	190	249	665
	DC98	47	116	270	97	407	956
	DC15	23	33	77	106	367	290
None (L-15)	EVO	<10	<10	<10	192	329	211
	DBV1	<10	<10	<10	85	137	249

^a PRNT₆₀, 60% plaque reduction neutralization titer. Limit of detection for neutralizing antibodies is 1:10. All monkeys were seronegative for LACV, TAHV, and JCV (PRNT, <10) on day 0 of inoculation.
^b All groups were challenged with 10⁵ PFU of JCV on day 42 following primary inoculation.

lated from dually infected mosquitoes and containing the full-length M segment from JCV in a LACV background was viable, with neuroinvasiveness and neurovirulence phenotypes like those of its JCV parent virus (9). This indicated that the JCV G_N, NS_M, and G_C proteins were compatible with the L, N, and NS_S proteins of LACV, thus increasing the likelihood that the desired rJCV/LACV antigenic chimeric virus could be obtained.

rLACV and rJCV/LACV were easily recovered from cDNA plasmids, with titers greater than 10⁶ PFU/ml, in as few as 2 to 3 days posttransfection. Chimerization did not affect virus growth in tissue culture, and rJCV/LACV maintained the robust growth kinetics observed for the parental viruses. The absence of *in vitro* growth restriction following chimerization was not surprising, since the reassortant virus that contained the full-length M segment from JCV in a LACV background exhibited virulence similar to that of its JCV parent in mice (9).

Chimeric rJCV/LACV is highly infectious for Swiss Webster mice, maintaining ID₅₀ values similar to those for the parental JCV virus after either i.c. or i.p. inoculation. Like the parental JCV virus, the rJCV/LACV virus is not neuroinvasive. However, unlike both JCV and LACV, which are fully neurovirulent, chimeric rJCV/LACV did not cause disease after i.c. inoculation, and it replicated in the mouse CNS to very low levels that were 1,000- to 10,000-fold lower than those for the wild-type parent viruses. In monkeys, infection with the chimeric virus resulted in a significantly lower level of viremia than that seen with wild-type JCV. Thus, chimeric rJCV/LACV has an unexpected host range attenuation phenotype in which it replicates efficiently in mammalian cells *in vitro* but is highly restricted in replication in the CNS of mice and in the peripheral tissues of rhesus monkeys.

The rJCV/LACV chimeric virus is attenuated despite the fact that the S and L genome segments are derived from wild-type LACV and do not contain attenuating mutations. The mechanism underlying this host range attenuation phenotype was not explored in the present study, but several observations suggest areas of future research. First, rJCV/LACV, but not the reassortant containing the full-length M segment from JCV in a LACV background, was attenuated *in vivo*. These two viruses differed in two respects: they had different LACV parental viruses, and they had different 3' and 5' ends of the M segment. The specific factor(s) that contributed to the attenuation of rJCV/LACV could result from host-dependent protein-protein interactions or to a host-dependent effect altering a function of the chimeric 3' and 5' ends. Chimerization has been useful to attenuate other viruses for use as live attenuated vaccines, with attenuation presumably coming from altered protein-protein interactions. Whitehead et al. created a recombinant dengue type 4 virus that contained the ME or CME proteins of dengue type 2 virus. The resulting chimeric viruses contained fully wild-type proteins, yet they were attenuated for mosquitoes, mice, and monkeys (31). Chimerization of West Nile virus and wild-type dengue type 4 virus also resulted in attenuation and a reduction in mouse neurovirulence compared to results for the West Nile virus parent (25). It is also possible that attenuation could come from a disrupted function contained in nucleotide sequences in the 3' or 5' UTR and their adjacent sequences in the M ORF. In influenza virus, packaging signals extend from the UTR into the adjacent coding region, a mechanism proposed to ensure that the majority of virions contain all eight genome segments (14). If the packaging sequences for bunyaviruses are structured similarly to those for influenza virus, the gen-

eration of the chimeric junctions of LACV UTRs and the JCV ORF could have interrupted a required LACV packaging signal that extended into the ORF. If this were indeed the case, the effect of this loss of packaging on replication would have to be much greater *in vivo* than *in vitro*.

We have previously demonstrated that JCV infection generates a neutralizing antibody response that also efficiently neutralizes both LACV and TAHV viruses (3, 4). In the current study, we found that immunization with rJCV/LACV protected mice against lethal challenge with JCV, LACV, or TAHV. In monkeys, infection with JCV, rLACV, or rJCV/LACV protected against challenge with wild-type JCV, even in the absence of detectable neutralizing antibodies. Thus, infection with a single virus, rJCV/LACV, is able to protect against three different members of the CEV serogroup, making rJCV/LACV an attractive vaccine candidate.

This study was a proof-of-concept characterization of a chimeric bunyavirus vaccine candidate. The chimeric virus has very promising phenotypes in mice (neither neurovirulent nor neuroinvasive) and in monkeys (decrease in viremia), and future work will need to examine the possibility of the generation of reassortant vaccine/wild-type viruses and the level of replication and pathology associated with the intracerebral inoculation of rhesus monkeys. Although it seems unlikely that a vaccine/wild-type reassortant virus could become more virulent than the wild-type parent already present, this concern could be avoided by vaccinating only during seasons with low mosquito activity and counseling vaccinees on methods to avoid mosquito exposure. We recognize the challenge of demonstrating vaccine safety in the absence of a nonhuman primate disease model, but we do believe future work utilizing i.c. inoculation of monkeys to measure replication in the CNS will be the best measure of virus attenuation in the CNS. If the virus still is able to replicate within the CNS, the S and L segments would be good targets to incorporate additional attenuating mutations, since the M segment has already been modified and resulted in a degree of attenuation. Additional attenuating mutations by modification of the S and L segments could also be a method to reduce the possibility of the generation of pathogenic reassortants. Only after the chimeric virus is observed to be highly attenuated for the monkey CNS could this vaccine candidate move closer to human trials.

Here, we describe the first recovery of a recombinant chimeric bunyavirus expressing proteins of a related CEV and demonstrate that a single genetic process in which a chimeric M segment was generated yielded a highly attenuated candidate vaccine virus without the necessity of introducing specific attenuating mutations in the S or L gene segment or the M segment ORF. If this process also serves to attenuate other bunyaviruses within the CEV serogroup or within other bunyavirus genera, it could be a valuable method for future bunyavirus vaccine development. Chimerization did not affect growth of the virus in tissue culture cells, yet the virus replicated poorly in the mouse CNS and achieved a significantly lower mean peak titer in monkeys. Future work will focus on evaluation of the mechanism of attenuation of the rJCV/LACV in mice and on its attenuation for the CNS of nonhuman primates after i.c. inoculation. In addition, the ability to generate other antigenic chimeric viruses on the LACV background will be explored to examine the usefulness of this approach to generate live attenuated vaccines for other pathogenic bunyaviruses.

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