Neuroattenuated Bunyavirus Variant: Derivation, Characterization, and Revertant Clones

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A neuroattenuated variant bunyavirus, designated RFC/25B.5 (B.5), was selected by serial passage of a reassortant clone (RFC virus) of a California serogroup virus in BHK-21 cells, followed by plaque purification of that passaged stock. Based on its virulence index (ratio of PFU/50% lethal dose), clone B.5 was over 40,000-fold less virulent than its unpassaged RFC parent after intracerebral (i.c.) inoculation into adult mice. Clone B.5 also exhibited markedly reduced neuroinvasiveness after subcutaneous injection into neonatal mice, although it retained its ability to replicate and kill suckling mice after i.c. injection. A murine neuroblastoma line (NA cells) can be used as an *in vitro* surrogate for the adult mouse brain, since clone B.5 replicated to at least 1,000-fold-lower titers in NA cells than did several neurovirulent California serogroup viruses. Clone B.5 replicated in BHK-21 cells at 37°C to titers similar to those achieved by other California serogroup viruses but was temperature sensitive (*ts*) since its replication was markedly restricted at 38.9°C. Ten *ts* revertant clones of B.5 virus were selected at 38.9°C, and all of them lost their *ts* phenotype and regained the ability to replicate to high titer in NA cells and to kill adult mice after i.c. injection. Clone B.5 is the first described California serogroup virus which is truly attenuated after i.c. inoculation, and its availability will permit genetic analysis of bunyavirus neurovirulence.

The California serogroup of bunyaviruses offers an attractive model to study the molecular determinants of viral virulence. La Crosse virus, a North American member of the serogroup, infects humans and usually causes an inapparent subclinical infection, although it may rarely initiate an acute encephalitis which is infrequently fatal (5, 5a, 6, 11). The same spectrum of illness and type of disease is produced in mice after infection with California serogroup viruses, and the outcome can be manipulated by altering virus dose, route of inoculation, and age of the host (7, 9).

After subcutaneous (s.c.) injection of mice, which mimics natural transmission by the mosquito vector, the virus replicates in striated muscle, produces a plasma viremia, invades the central nervous system (CNS), and replicates in brain and spinal cord where it causes an acute cytotoxic infection of neurons and glia (7, 9). This sequence of events can be divided into neuroinvasiveness and neurovirulence.

Neuroinvasiveness is a measure of the ability of the virus to invade the CNS after peripheral injection and may be assessed by s.c. injection of suckling mice and quantitated as the ratio PFU/50% lethal dose (LD₅₀). Most California serogroup viruses are neuroinvasive and, after s.c. injection of suckling mice, replicate in striated muscle and produce a high-titer viremia. Nonneuroinvasive strains exhibit restricted replication in peripheral tissues and fail to invade the CNS (7). The trisegmented genome of bunyaviruses has permitted construction of reassortants between neuroinvasive and nonneuroinvasive California serogroup viruses, and genetic studies have shown that neuroinvasiveness is mainly determined by the middle-sized (M) genome segment and its products (4, 8, 18, 19).

Neurovirulence denotes the ability of the virus to infect the central nervous system and produce disease and is quantitated by intracerebral (i.c.) titration of virus and computation of the ratio PFU/LD₅₀. In the California serogroup, viremia does not occur after i.c. injection of adult mice (7, 9), and this method of challenge can be used to distinguish neurovirulence from neuroinvasiveness. All natural isolates of California serogroup viruses are neurovirulent, i.e., the PFU/LD₅₀ ratio is about 1 (5, 5a, 19), and the lack of a highly neuroattenuated virus clone has precluded studies of the genetics of bunyavirus neurovirulence. We now report the selection of a neuroattenuated clone (RFC/25B.5) (designated B.5) of California serogroup virus, characterize its pathogenesis in mice, and show that its attenuation is reflected in its inability to replicate in mouse neuroblastoma cells (clone NA). Furthermore, we show that clone B.5 exhibited a temperature-sensitive (*ts*) phenotype and that *ts* revertant clones regained the neurovirulence phenotype of the parent virus from which B.5 was derived.

MATERIALS AND METHODS

Animals. Outbred albino Swiss mice (CD-1; Charles River Breeding Laboratories, Inc., Portage, Mich.) were used in all experiments.

Viruses and cells. The origin of both La Crosse/original (LAC) and Tahyna/181-57 (TAH) viruses has been previously described (7). RFC virus is a reassortant between LAC and TAH viruses and was obtained by passaging the previously described P1-13c4 virus (8) one additional time on BHK-21 cells. LAC/PP-31, a plaque-purified variant of LAC virus (13), was passaged two additional times on BHK-21 cells before use in this study. Another variant of LAC virus, LAC/p10 virus, was obtained after LAC virus had been

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passed an additional 10 times on BHK-21 cells. Working stocks of all viruses were prepared as clarified tissue culture supernatants from infected BHK-21 cells. Viral harvests were aliquoted and frozen at -80°C before use.

BHK-21 cells were propagated as previously described (7). NA cells, originally derived from the murine neuroblastoma cell line C-1300 (12), were maintained in Eagle minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS), 100 U of penicillin per ml, and 100 μg of streptomycin per ml.

Isolation of neuroattenuated variant. Duplicate samples of five different virus strains (LAC, LAC/PP-31, LAC/p10, TAH, and RFC) were subjected to serial passage on monolayers of BHK-21 cells. Viruses diluted in an infection medium consisting of MEM supplemented with 2% FCS, 293 μg of glutamine per ml, 100 U of penicillin per ml, and 100 μg of streptomycin per ml were used to infect confluent monolayers of 2×10^5 BHK-21 cells grown in 24-well plates. The initial multiplicity of infection for each virus was 0.01 PFU per cell. After 2 h at 35°C , supernatants containing desorbed virus were removed and monolayers were washed three times with MEM containing 2% FCS. Fresh medium (1 ml) was added to each well, and cultures were incubated for an additional 22 h at 35°C . Supernatants were harvested, clarified by centrifugation ($1,000 \times g$), and used undiluted for the next cycle of infection. After 25 passages on BHK-21 cells, working stocks were aliquoted and frozen at -80°C . Virus titers for each of the working stocks were determined by a standard plaque assay on BHK-21 cells (7).

Determination of viral virulence. For quantitation of neuroinvasiveness, 0.05 ml of $\log_{10}$ dilutions of selected viruses was inoculated s.c. into 3-day-old suckling mice (one litter per dilution). Neurovirulence was quantitated by i.c. injection of 0.02 ml of virus dilutions into either adult mice (five mice per $\log_{10}$ dilution) or 3-day-old suckling mice (one litter per $\log_{10}$ dilution). In all cases, mortality was recorded for 14 days and LD_{50} values were computed by the method of Reed and Muench (16). Titers were also determined by plaque titration of viral dilutions on BHK-21 cells, and virulence was recorded as the ratio of PFU/ LD_{50} .

Tissue titrations. To examine the replication of select viruses in mice, we performed tissue titrations as previously described (7). Briefly, infected adult and suckling mice were sacrificed, and selected tissues (brain and blood) were harvested, pooled, and prepared as 10% suspensions in MEM and 2% FCS. Viral titers were determined by plaque titration on BHK-21 monolayers and expressed as PFU per milligram of brain or microliter of plasma.

Immunofluorescence observations. Mouse brains were harvested and frozen at -80°C . Parasagittal sections were cut at 6 μm in a cryostat, air dried, fixed in cold acetone, and stored at -20°C until examination. An indirect immunofluorescence technique was used (7). The primary and secondary antibodies were, respectively, a polyclonal rabbit anti-LAC virus antiserum and a fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (Organon Teknika, West Chester, Pa.). Evan blue was added to the conjugate as a counterstain (0.5% final concentration), and sections were examined by epi-illumination in a Zeiss photomicroscope.

Immunofluorescence studies on cultured cells were performed with a combination of monoclonal antibodies directed against G1 and N of LAC and TAH. The cells were grown on Lab-Tek chamber slides (Nunc, Naperville, Ill.), infected with the appropriate virus at a multiplicity of infection of 0.1, and fixed 24 h after infection by brief (1- to

TABLE 1. Neurovirulence of 10-passage stocks of La Crosse and TAH viruses

Parent virus	Genotype	Passage series	Mortality after i.c. injection of 100 PFU in adult mice (no. dead/total tested)
LAC	LLL	25A	5/5
		25B	3/5
LAC/p10	LLL	25A	3/5
		25B	4/5
LAC/PP-31	LLL	25A	5/5
		25B	4/5
TAH	TTT	25A	5/5
		25B	5/5
RFC	TLL	25A	1/5
		25B	0/5

2-min) exposure to 3% paraformaldehyde. The cells were washed and postfixed with cold acid-alcohol (95% ethanol, 5% acetic acid). The cells were then washed and treated with phosphate-buffered saline containing 20% goat serum, exposed to the primary antibody, washed, and then exposed to fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (Organon Teknika, West Chester, Pa.).

Growth curves. Monolayers of BHK-21 and NA cells grown in 24-well plates were infected at a multiplicity of infection of 0.01, rocked for 30 min at room temperature, washed three times with MEM containing 2% FCS, and incubated at either 37 or 39.8°C . At various times after infection, supernatants were harvested and clarified by centrifugation (4) and their titers were determined by plaque assay on BHK-21 cells (7).

Preparation of *ts* revertants. Ten *ts* revertants of the neuroattenuated RFC/25B.5 variant were obtained by plaqueing on BHK-21 monolayers at the restrictive temperature of 39.8°C . Plaques were picked and purified by two further plaque passages, and working stocks were prepared from infected BHK-21 cells maintained at 39.8°C . The *ts* phenotype of the revertants was examined by plaque titration on BHK-21 cells at 37 and 39.8°C .

RESULTS

Isolation of neuroattenuated variant virus. Five viruses (LAC, LAC/PP-31, LAC/p10, TAH, and RFC, each in duplicate) were passaged 25 times in BHK-21 cells in an attempt to select a neuroattenuated California serogroup variant virus. The reassortant RFC virus (containing a TAH virus L and LAC virus M and S segments) was selected for passage because it showed an unexpectedly low peripheral virulence ratio (8). In addition to this, it was noted to have a slight change in the mobility of its nucleocapsid protein (data not shown). To assess the neurovirulence of these passaged virus stocks, adult mice were inoculated i.c. with 100 PFU of virus (Table 1).

The most neuroattenuated of these 10 passaged virus stocks, RFC/25B, produced no mortality and was selected for further plaque purification. Ten clones of RFC/25B were triple plaque purified on BHK-21 cells, and adult mice were injected i.c. with 10,000 PFU. Mortality varied from 0 to

TABLE 2. Neurovirulence of 10 clones of RFC/25B virus

Clone	Mortality after i.c. injection of 10,000 PFU in adult mice (no. dead/total tested)
1	5/5
2	4/5
3	5/5
4	3/5
5	0/5
6	4/5
7	2/5
8	1/5
9	5/5
10	1/5

100% (Table 2), and the most neuroattenuated of the clones, RFC/25B.5 (designated B.5), was selected for further characterization.

i.c. infection of adult mice. To compare clone B.5 with selected members of the California serogroup, viruses were titrated i.c. in adult mice and virulence was expressed as the $\log_{10}$ of the ratio PFU/LD₅₀ (Table 3). B.5 was about 40,000-fold less virulent than its parent, RFC virus, and more than 1,000,000-fold less virulent than LAC virus and the reassortant virus P1-2gg (another reassortant of genotype TLL). Therefore, it is unlikely that the TLL genotype per se is responsible for the marked attenuation exhibited by clone B.5.

To examine viral replication in the CNS, we injected adult mice i.c. with 700 PFU of B.5 virus, LAC virus, TAH virus, and RFC virus (Fig. 1). Mice infected with TAH, LAC, or RFC virus were moribund by 4, 5, and 10 days, respectively, while mice infected with B.5 virus remained asymptomatic. B.5 virus barely replicated, never exceeding 1 PFU/mg, while the other viruses reached levels from 10⁴ to 10⁶ PFU/mg in moribund animals. A similar picture emerged when adult mice were injected i.c. with an inoculum of 500,000 PFU (data not shown).

To localize B.5 virus replication within the CNS, we used immunofluorescent staining of viral antigen. Specific fluorescence was not seen in the brains of mice infected with B.5, unlike those infected with LAC, which show viral antigen, particularly in neurons (7).

i.c. infection of suckling mice. To examine the effect of host age on the neurovirulence phenotype, we titrated selected

TABLE 3. Virulence of RFC/25B.5 virus compared with selected strains of LAC and TAH viruses^a

Virus strain	Genotype	i.c. infection of adult mice	i.c. infection of suckling mice	s.c. infection of suckling mice	NA cells ^b
LAC	LLL	0.1	-0.4	-0.3	-0.3
TAH	TTT	0	-0.5	4.2	-0.3
Reassortant P1-2gg	TLL	0.3	0.2	0.1	0
Reassortant RFC	TLL	1.5	-0.1	1.4	0.2
Reassortant RFC/25B.5	TLL	>6.1	0.4	5.4	6.2

^a Virulence expressed as $\log_{10}$ of the ratio PFU (BHK-21 cells)/LD₅₀ (mice).

^b NA phenotype expressed as $\log_{10}$ of the ratio PFU (BHK-21 cells)/PFU (NA cells). PFU were assayed at 37°C.

FIG. 1. Replication of LAC (○), TAH (●), RFC (■), and RFC/25B.5 (□) viruses in adult mice after the i.c. injection of 700 PFU of each virus. The wild-type LAC and TAH viruses and the parent reassortant RFC replicated to high titer, while the neuroattenuated strain RFC/25B.5 replicated poorly.

California serogroup viruses i.c. in suckling mice (Table 3). Clone B.5 was almost as neurovirulent in suckling mice (2 to 3 PFU/LD₅₀) as other California serogroup viruses (about 1 PFU/LD₅₀), reflecting the age dependence of host susceptibility (5).

s.c. infection of suckling mice. The neuroinvasiveness of selected California serogroup viruses was determined by s.c. titration of virus in suckling mice (Table 3). B.5 virus exhibited low neuroinvasiveness (>100,000 PFU/LD₅₀) which is clearly associated with the extraneural phase of infection, since this virus is virulent on direct i.c. injection in suckling mice. RFC virus, the parent of clone B.5, showed a slight reduction in neuroinvasiveness (30 PFU/LD₅₀) compared with virulent strains such as LAC virus (<1 PFU/LD₅₀). TAH virus exhibited reduced neuroinvasiveness but high neurovirulence as reported previously (7, 8).

To further determine the basis of the reduced neuroinvasiveness of clone B.5, suckling mice were injected with 700 PFU of B.5 virus or RFC virus and selected tissues (brain and blood) were tested at intervals (Fig. 2). B.5 virus could not be detected at any time after infection, while RFC virus produced a viremia and invaded the brain where it replicated to high titer.

FIG. 2. Replication in blood and brain after the s.c. injection of 700 PFU of the neuroattenuated clone RFC/25B.5 compared with replication of the parent reassortant, RFC. Symbols: ■—■, RFC in brain; ■---■, RFC in blood; □, B.5.

FIG. 3. Replication in NA cells after infection at a multiplicity of 0.01 of the neuroattenuated clone RFC/25B.5 (□) compared with RFC (■), LAC (○), TAH (●), and reassortant P1-2gg (◇).

Replication in NA cells. Infection of NA cells, a murine neuroblastoma cell line, was examined to identify an *in vitro* surrogate for the adult mouse brain (2). Clone B.5 barely replicated (maximum titer, $10^{2.3}$ PFU/ml) (Fig. 3), while LAC and TAH viruses reached titers of 10^7 PFU/ml, similar to those seen in infected BHK cells. B.5 virus caused little detectable cytopathic effect in NA cells, while LAC and TAH viruses destroyed the monolayers in 24 to 48 h. When these viruses were plaqued on NA cells and the titers compared with those obtained on BHK cells, the ratio of titers (BHK/NA) was $>10^6$ for B.5 virus but close to 1 for the other viruses tested (Table 3).

To further confirm that growth in NA cells was associated with the neurovirulence phenotype and not the genotype TLL, we also determined growth curves with neurovirulent reassortant P1-2gg (genotype TLL [8]) and determined that its growth characteristics in NA cells were similar to those of LAC and TAH viruses (Fig. 3).

Immunofluorescence. A combination of monoclonal antibodies directed against G1 and N was then used to assess the pattern of antigen expression in NA cells infected with LAC, RFC, and B.5 viruses. LAC caused a widespread infection of the cultured monolayer, with expression of viral antigens in $>95\%$ of the fixed culture (Fig. 4A). RFC virus showed a diminution in immunofluorescence (Fig. 4C). NA cells infected with B.5 showed minimal fluorescence in scattered cells (Fig. 4D). Cells infected with the reassortant P1-2gg resembled those infected with wild-type LAC in the pattern of antigen expression (Fig. 4B).

Replication in BHK-21 cells. The kinetics of replication of B.5 virus were compared with those of LAC, TAH, and RFC viruses on BHK-21 cells at 37°C (Fig. 5, upper panel). B.5 reached slightly lower titers than the other viruses (2- to 10-fold lower) but replicated at about the same rate. Immunofluorescence studies showed that BHK-21 cells infected with B.5 expressed antigens throughout the monolayer (Fig. 4F). The perinuclear localization of the viral antigens was more marked in the cultures infected with B.5.

When replication in BHK-21 cells was determined at 39.8°C , a different picture emerged (Fig. 5, lower panel). Clone B.5 displayed a dramatic *ts* phenotype (titer of $<10^{1.7}$ PFU/ml), while LAC virus grew to high titer ($>10^8$ PFU/ml), and RFC virus displayed an intermediate *ts* phenotype (maximum titer, 10^5 PFU/ml).

***ts* revertants of clone B.5.** To obtain *ts* revertants, we plaqued B.5 virus on BHK-21 cells at 39.8°C , and 10 plaques

were picked and further plaque purified. The 10 clones were tested for *ts* phenotype by comparing plaque titers at permissive and restrictive temperatures (Table 4). All 10 clones exhibited a ratio of plaque titers ($37^\circ\text{C}/39.8^\circ\text{C}$) of about 10, compared with a ratio of about 10^5 for clone B.5 and about 1 for LAC virus. Thus, all 10 clones were *ts* revertants.

The *ts* revertant clones were plaqued on NA cells at 37°C , and the ratio of plaque titers (BHK-21/NA) was determined (Table 4). All 10 clones showed a revertant NA phenotype (ratios about 10) compared with a ratio of $>10^6$ for clone B.5 and a ratio of 1 for LAC virus. To confirm the validity of the NA cell model, the *ts* revertant clones were further tested for neurovirulence by *i.c.* injection of adult mice of 100 and 10,000 PFU. All the *ts* revertants appeared to have markedly increased neurovirulence. Two revertants, *ts*20 and *ts*25 (Table 4) were tested more extensively. They exhibited markedly increased neurovirulence (PFU/LD₅₀ ratios of $10^{1.6}$ and $10^{2.3}$, respectively) compared with clone B.5 ($>10^6$ PFU/LD₅₀) and had titers similar to that of parent RFC virus (Table 4).

DISCUSSION

Neurovirulence and its further analysis. Because of their segmented genome and capacity for reassortment, the California serogroup viruses offer an excellent system for analysis of the genetic determinants of defined biological properties. We have previously used this model to map neuroinvasiveness to the M RNA segment (4, 7, 8). However, analyzing neurovirulence with reassortants has been impossible because all the serogroup members are highly virulent when injected *i.c.* In this report, we described the first highly neuroattenuated California serogroup virus. Another attenuated virus clone (designated I-16) (17) has been studied, but this strain was only 4- to 10-fold less virulent than a comparable virulent clone. In contrast, PFU/LD₅₀ ratios indicated that clone B.5 is more than 40,000-fold less virulent than either of the parent viruses or other reassortants with the same genotype. The striking neuroattenuation of clone B.5 will permit genetic analysis of neurovirulence. For example, crosses between clone B.5 (genotype TLL) and a neurovirulent reassortant (B1-1a, genotype LTT) of TAH and LAC viruses should define the gene segment(s) responsible for the neuroattenuated phenotype of clone B.5.

In addition to constructing reassortant viruses, we were able to exploit the *ts* phenotype of this virus to select virulent revertants. The *ts* revertants occurred at a frequency of about 10^{-5} , suggesting that the revertant phenotype is due to a single point mutation. Since the *ts* phenotype is associated with increased virulence, sequencing the appropriate gene segment should reveal mutations associated with attenuation.

Rapid replication in cell culture and attenuation in mice. Previous work with the alphaviruses has demonstrated that clones selected for rapid penetration of BHK-21 cells are attenuated after peripheral inoculation of mice (1, 10, 14, 15). We used a passage protocol which selected for rapidly growing (and presumably early penetrating) virus. Nevertheless, our results did not suggest that rapid penetration was related to selection of an attenuated clone of California serogroup virus, since only the passaged RFC virus showed reduced neurovirulence among the strains examined.

***ts* phenotype and neuroattenuation of clone B.5.** It appears likely that temperature sensitivity and neuroattenuation are pleiotropic effects of one or several mutations in the viral genome and that reduced virulence is not a secondary

FIG. 4. Expression of viral antigens after infection of NA cells (A to D) and BHK-21 cells (E and F). Infection of NA cells with LAC (A) and reassortant P1-2gg (B) resulted in abundant expression of viral antigens as detected by indirect immunofluorescence with monoclonal antibodies directed against G1 and N (see Materials and Methods). RFC virus demonstrated some decrease in fluorescence (C), and infection with the neuroattenuated virus RFC/25B.5 (D) resulted in minimal antigen expression. Infection of BHK-21 cells with either LAC virus (E) or the neuroattenuated virus RFC/25B.5 (F) demonstrated similar perinuclear accumulation of viral antigens.

consequence of the *ts* phenotype. Replication of B.5 virus is restricted in mice even though the body temperature of the mouse is 37°C (20), which is a permissive temperature for clone B.5 in BHK-21 cells (Fig. 5). Likewise, clone B.5 is restricted in NA cells at 37°C (Fig. 3). Finally, in preliminary studies, B.5 appears to have lost its neurotropism in mosquitoes which are maintained at room temperature (22°C) (Barry J. Beaty et al., personal communication).

NA cells as cell culture surrogate for in vivo neurovirulence. NA cells were derived from a murine neuroblastoma and express many neuronal markers (12). Previous studies have shown that passage through NA cells can select virulent rabies virus from a neuroattenuated (HEP) strain (2). Furthermore, Dietzschold and co-workers (3) could detect differences in cell-to-cell spread between an avirulent and

virulent strain of rabies virus in these cells. Therefore, the NA cell line demonstrates differences between viruses that relate to neuronal infection and can serve as a surrogate for the mouse brain.

When we compared our neuroattenuated B.5 clone with its virulent parents, we saw even more dramatic differences in replication than were seen with neuroattenuated rabies virus, since the yield of cell-free virus in NA cells was over 10,000-fold lower for attenuated B.5 than for LAC or TAH viruses. This was reflected in the ratio of PFU titers in BHK-21 cells compared with those in NA cells; this ratio was over 100,000 for clone B.5 and about 1 for LAC virus. It appears that NA cells can be used as a convenient surrogate for rapid screening of California serogroup viruses for potential neuroattenuation and perhaps for the selection of

FIG. 5. Replication in BHK-21 cells of four viruses at 37 and 39.8°C after infection at a multiplicity of 0.01. The neuroattenuated virus RFC/25B.5 (□) demonstrated marked temperature sensitivity with minimal replication at 39.8°C (bottom panel). At 37°C (top panel), replication in BHK-21 cells was equivalent to that of LAC (○), TAH (●), and RFC (■) viruses.

revertant neurovirulent clones of B.5 virus. Furthermore, these cells may provide a powerful system to determine the step(s) in replication which distinguishes neuroattenuated and neurovirulent clones.

TABLE 4. *ts* revertants (*tsr*) of a neuroattenuated clone (B.5) of California serogroup bunyavirus

Virus	Geno- type	<i>ts</i> phenotype in BHK-21 cells (log PFU 33°C/40°C)	NA phenotype (log PFU BHK-21 cells/ NA cells)	i.c. virulence in adult mice (log PFU/LD ₅₀)
B.5	TLL	5.3	6.2	>6.1
<i>tsr</i> 17	TLL	1.1	1.2	
<i>tsr</i> 18	TLL	1.1	1.3	
<i>tsr</i> 19	TLL	1.2	1.3	
<i>tsr</i> 20	TLL	0.9	1.4	1.6
<i>tsr</i> 21	TLL	1.0	1.3	
<i>tsr</i> 22	TLL	0.9	1.2	
<i>tsr</i> 23	TLL	0.7	1.1	
<i>tsr</i> 24	TLL	1.2	1.5	
<i>tsr</i> 25	TLL	1.4	1.2	2.3
<i>tsr</i> 26	TLL	0.8	1.1	
RFC	TLL	2.6	0.2	1.5
LAC	LLL	0.0	-0.2	0.1

The neuroattenuated strain B.5 will provide a useful tool to study the genetics of bunyavirus neurovirulence and will likely provide insights into the molecular mechanisms of neuronal infection. The NA cells should prove to be a useful model to study the interactions between virus and cells of neuronal origin at a molecular and biochemical level.

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