Neuroattenuation of an Avirulent Bunyavirus Variant Maps to the L RNA Segment

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The derivation and characterization of a neuroattenuated reassortant clone (RFC 25/B.5) of California serogroup bunyavirus was described previously (M. J. Endres, A. Valsamakis, F. Gonzalez-Scarano, and N. Nathanson, *J. Virol.* 64:1927-1933, 1990). To map the RNA segment responsible for this attenuation, a panel of reassortants was constructed between the attenuated clone B.5 (genotype TLL) and a virulent clone (B1-1a) of reciprocal genotype (LTT). Parent viruses and clones representing all of the six possible reassortants were examined for neurovirulence by intracerebral injection in adult mice. Reassortants bearing the large RNA segment from the virulent parent were almost as virulent as the virulent parent virus, while reassortants bearing the large RNA segment from the avirulent parent virus exhibited low or intermediate virulence. These results indicate that the large RNA segment is the major determinant of neuroattenuation of clone B.5. In addition to its neuroattenuation, clone B.5 was temperature sensitive and exhibited an altered plaque morphology. These phenotypes also segregated with the large RNA segment. The importance of the large RNA segment (which encodes the viral polymerase) in neurovirulence contrasts with prior studies which indicate that the ability to cause lethal encephalitis after peripheral injection of suckling mice (neuroinvasiveness) is primarily determined by the middle-sized RNA segment, which encodes the viral glycoproteins.

To study the molecular determinants of viral virulence and neurotropism, we have focused on La Crosse virus, a member of the California serogroup of the family *Bunyaviridae*. Bunyavirus genomes are composed of three separate single-stranded RNA segments designated large (L), middle-sized (M), and small (S). For members of the *Bunyavirus* genus, all three segments are of negative polarity (27). The L RNA segment encodes the L protein, presumed to be the viral polymerase (4, 5); the M segment codes for the two structural glycoproteins (G1 and G2) and at least one nonstructural protein designated NS_m (7); and the S RNA segment codes for the nucleocapsid protein (N) and the NS_s nonstructural protein (2, 9, 10). The trisegmented nature of the bunyavirus genome has permitted the construction of reassortant viruses between California serogroup viruses having different phenotypes. Reassortants have been used to map distinct phenotypes to specific gene segments, including electrophoretic mobilities of viral proteins, temperature sensitivity, plaque morphology, viral tropism, and viral virulence.

La Crosse virus and some other members of the California serogroup are associated with cases of human encephalitis in North America (14, 18, 23, 30, 32). La Crosse virus infection of mice offers a good model system for the human disease and may be used to address questions concerning viral virulence and pathogenesis (15, 17). In mice, the outcome of infection is dependent on the strain of virus, virus dose, route of infection, and age of the mice, variables which can be manipulated experimentally. The course of infection may be divided into two major phases, neuroinvasiveness and neurovirulence.

Neuroinvasiveness is a measure of the ability of a virus to invade the central nervous system (CNS) and produce

disease after peripheral inoculation and encompasses the early stages of a natural infection, i.e., viral replication in extraneural tissues (primarily striated muscle), production of a plasma viremia, and spread to the CNS. Most California serogroup viruses are neuroinvasive, and those which are not exhibit restricted replication in peripheral tissues and consequently fail to invade the CNS (6, 15). Genetic studies using reassortants show that neuroinvasiveness is primarily determined by the M RNA segment (11, 16, 28). Furthermore, experiments with monoclonal antibody-resistant variant viruses implicate the G1 glycoprotein (which is encoded by the M RNA segment) in neuroinvasiveness (12).

Neurovirulence, on the other hand, reflects the ability of a virus to replicate and produce disease in the CNS and can be quantitated as the ratio of PFU to 50% lethal doses (LD₅₀) after intracerebral (i.c.) inoculation of adult mice. Adult mice injected i.c. with California serogroup viruses fail to develop a plasma viremia, and viral replication is restricted to the CNS (15, 17). Therefore, i.c. challenge of adult mice constitutes an evaluation of neurovirulence uncomplicated by extraneural virus replication. All natural isolates of California serogroup viruses are highly neurovirulent, with a PFU/LD₅₀ ratio of about 1 (13, 14). A few neuroattenuated laboratory clones of California serogroup viruses have been described (25), but these exhibited only minimal reduction in virulence.

Clone RFC/25 B.5 (B.5), derived by passage in BHK-21 cells, is the first highly neuroattenuated California serogroup virus to be described; it has a PFU/LD₅₀ ratio of greater than 10⁶ (6). Other salient characteristics of B.5 virus include its TLL genotype (the three-letter code refers to the origin of the L, M, and S RNA segments, respectively, with L for La Crosse and T for Tahyna virus), its temperature sensitivity at 39.8°C, and its lack of neuroinvasiveness in neonatal mice.

In this study, we genetically crossed B.5 virus with a neurovirulent reassortant virus (clone B1-1a) possessing an

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LTT genotype (16). Progeny reassortant viruses were then examined with respect to neurovirulence and several other phenotypes. Our results indicate that the L RNA segment is a major determinant of the neuroattenuation of clone B.5, while the M RNA segment has no influence on neurovirulence, in contrast to its importance as the major determinant of neuroinvasiveness.

MATERIALS AND METHODS

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

Viruses and cell cultures. The origin and characterization of the neuroattenuated B.5 virus and the neurovirulent B1-1a virus have been previously described (6, 16). Clone B.5 is a neuroattenuated variant virus, derived by 25 passages of a reassortant virus (genotype TLL) through BHK-21 cells, followed by plaque purification of the passaged stock. Clone B1-1a is a neurovirulent plaque-purified recombinant clone (genotype LTT) derived from a cross between La Crosse/ original and Tahyna/181-57 viruses and has an LTT genotype. Working stocks of both viruses were prepared as clarified tissue culture supernatants from BHK-21 cells infected with plaque-purified virus. Viral harvests were aliquoted and frozen at -80°C before use.

BHK-21 cells are a continuous line of hamster fibroblasts, 3T3 cells are a line of fibroblasts derived from the NIH strain of mouse, L929 cells are a fibroblast cell line derived from a C3H mouse, NA cells are a clone of the C-1300 neuroblastoma mouse cell line (21), and C2C12 cells are a subclone of a C3H mouse myoblast cell line (1a, 37). BHK-21 and NA cells were propagated as previously described (6, 15).

Plaque morphology and temperature sensitivity. Viruses were plaqued according to published protocols (6, 15, 16). Medium was removed from subconfluent monolayers in six-well plates, and 0.2 ml of virus inoculum was added per well. After cultures were incubated at room temperature for 1 h on a rocker, an overlay consisting of 3 ml of 0.5% agarose in Eagle's minimum essential medium (MEM) with 2% fetal calf serum was added. Plaques were read after 3 days at 33 or 39.8°C in BHK-21 cells and after 3 or 5 days at 33°C in NA cells. Plaques were visualized by removing the agarose overlay and staining the remaining monolayer with amido black stain.

Preparation of reassortant viruses. Reassortant viruses were obtained by using a modified version of a published protocol (25). Mixtures containing various concentrations of both B.5 and B1-1a viruses were precipitated overnight at 4°C in the presence of polyethylene glycol (7.1%, wt/vol) and 0.4 M NaCl. Viruses were pelleted by centrifugation at $10,000 \times g$ for 30 min and resuspended in MEM containing 2% fetal bovine serum, 100 U of penicillin per ml, and 100 µg of streptomycin per ml (infection medium). Confluent monolayers of BHK-21 cells that had been grown in 24-well plates (approximately 2×10^5 cells per well) were inoculated with 0.1 ml of resuspended virus mixture per well. Multiplicities of infection for each parent virus ranged from 1 to 200 PFU per cell, and parent viruses were not always present in equal amounts. After 2 h at 33°C, the supernatants were removed and the monolayers were washed extensively with MEM, refed with infection medium, and incubated at 33°C for an additional 16 to 24 h. Supernatants were harvested, clarified, and stored at -80°C . After three direct plaque purifications of these supernatants, individual virus clones were obtained

TABLE 1. Comparison of virulence between attenuated clone B.5 and selected strains of La Crosse and Tahyna viruses^a

Virus strain ^b	Genotype ^c	i.c. virulence in ^d :		s.c. virulence in suckling mice ^d	Temperature sensitivity in BHK cells ^e
		Adult mice	Suckling mice		
La Crosse/original	LLL	0.1	−0.4	−0.3	0.0
Tahyna/181-57	TTT	0.0	−0.5	4.2	0.2
Reassortants					
P1-2gg	TLL	0.3 ^f	0.2	0.1	0.0
B.5	TLL	>6.1	0.4	5.4	5.3
B1-1a	LTT	0.0	0.3	3.9	0.0

^a Some of these data have been published elsewhere (6).

^b La Crosse/original and Tahyna/181-57 are prototype strains used for construction of reassortants.

^c See text for explanation.

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

^e Expressed as the $\log_{10}$ of the ratio PFU (33°C)/PFU (39.8°C).

^f LD₅₀ titration was done in weanling mice.

(16). Virus working stocks were then prepared as clarified tissue culture supernatants from infected BHK-21 cells.

Determination of viral genotypes. Triple-plaque-purified virus clones were genotyped by using a dot blot hybridization protocol previously described (5). Three La Crosse virus cDNAs, each of which was specific for either the L, M, or S RNA segment by Northern (RNA) analysis, were labeled with ³²P-nucleotides by the random priming method (8) and used to probe viral RNA immobilized on nitrocellulose. The source of viral RNA was total cytoplasmic RNA obtained from infected BHK-21 cell lysates and was prepared according to established methods (36). After the nitrocellulose filters were hybridized with segment-specific radiolabeled probes, washing conditions were selected such that labeled probes bound selectively to La Crosse virus-derived RNA and not to that which originated from Tahyna virus.

Determination of viral phenotypes. The methods used for phenotyping reassortant viruses by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) have been described elsewhere (5, 16). Viral proteins were labeled with [³⁵S]methionine and -cysteine and were subjected to SDS-PAGE (19) on 10% polyacrylamide gels. The gels were then treated with En³Hance (New England Nuclear Corp., Boston, Mass.), dried, and autoradiographed.

Determination of viral virulence. Viral neurovirulence was determined in mice as previously described (6). After adult mice were injected i.c. with 0.02 ml of virus dilution (five mice per 10-fold dilution), mortality was recorded for up to 21 days, and LD₅₀ values were computed (22). To compare the pathogenesis of different virus clones, 0.02 ml containing 700 PFU was injected i.c. into adult mice, animals were sacrificed at intervals, and brains were homogenized and tested for virus titer (15).

RESULTS

Comparison of attenuated clone B.5 with selected California serogroup viruses. As background information, attenuated clone B.5 is compared in Table 1 with selected California serogroup viruses, according to virulence profile and temperature sensitivity. La Crosse/original virus is the prototype virulent wild-type clone, which is highly virulent in both adult and suckling mice. By contrast, Tahyna/181-57 is

highly virulent by the i.c. route but shows markedly reduced neuroinvasiveness after subcutaneous (s.c.) injection in suckling mice. Clone P1-2gg is a reassortant, of genotype TLL, between La Crosse/original and Tahyna/181-57 viruses and is as neuroinvasive as La Crosse/original, since its M RNA segment was derived from La Crosse virus (16). Attenuated clone B.5 is also a reassortant of La Crosse/original and Tahyna/181-57 viruses of genotype TLL, but it is highly attenuated, both on i.c. injection of adult mice and on s.c. injection of suckling mice, reflecting the fact that it was passed 25 times in BHK-21 cells and selected for extreme attenuation (6). Thus, the attenuation of clone B.5 is inconsistent with the virulence of the parent viruses from which it was derived, implying that it has acquired biologically significant mutations in one or more segments of its genome. This study was designed to identify which segment(s) was responsible for attenuation, by crossing clone B.5 with a neurovirulent reassortant of reciprocal genotype. Clone B1-1a, of genotype LTT, was selected for this purpose, since it was highly neurovirulent (Table 1).

Construction and selection of reassortant viruses. Clones of B.5 (TLL genotype) and B1-1a (LTT genotype) viruses were used to coinfect BHK-21 cells. The plaque-purified progeny clones from these coinfections were genotyped by dot blot hybridization of viral RNA with three different 32 P-labeled La Crosse virus cDNAs, each of which was specific for one of three viral RNA segments. Viral RNAs originating from La Crosse virus bound labeled probe, whereas Tahyna virus-derived RNAs failed to do so with the stringency of washes used.

The rates of reassortment were found to vary considerably among different coinfections, ranging from 0 to 27% (data not shown). Among those coinfections which yielded any reassortants, the frequency of reassortants was 21% (77 of 370), much lower than the 75% which would be expected assuming unrestricted segment reassortment. Among the six possible reassortant genotypes, there was considerable variation in relative frequency without any discernible pattern. There was only one reassortant of genotype TLT. To obtain further reassortants of this genotype, 148 additional clones were phenotyped, of which one was found with an XLT phenotype; on genotyping this proved to be another TLT virus.

Viruses of the LLT genotype were not observed in any of the initial coinfections, and a second round of coinfections was therefore performed by backcrossing an LLL reassortant clone (J18/8 virus) with B1-1a virus (LTT genotype). Plaque-purified progeny viruses were then phenotyped by SDS-PAGE of 35 S-labeled viral proteins. Phenotyping gene products of the M segment (G1 protein) and the S segment (N protein) was sufficient to obtain complete viral genotypes, since both parental viruses possessed a La Crosse virus-derived L RNA segment. Among 26 clones tested (data not shown), 13 were reassortants, including 8 with an LLT genotype.

Neurovirulence of reassortant viruses. To determine the neurovirulence of reassortant viruses, we titrated them i.c. in adult mice and computed the ratios of PFU/LD₅₀ at 14 days after infection (Fig. 1). For convenience, genotypes are designated AAA for the segments derived from attenuated clone B.5 and VVV for segments derived from virulent clone B1-1a.

For the virulent B1-1a and the attenuated B.5 parental viruses, the log₁₀ ratios of PFU/LD₅₀ were 0.0 and >6.1, respectively, a difference in neurovirulence of more than 10⁶-fold. Among mice dying after injection with virulent

FIG. 1. Neurovirulence of virulent clone B1-1a, attenuated clone B.5, and reassortants between them. The virulence genotype follows the nomenclature used in Table 2, as defined in the text. Virulence is quantified as the log₁₀ of the ratio PFU/LD₅₀.

B1-1a virus, the survival time ranged from 6 to 9 days, depending on the dilution of virus. The few animals dying after injection of large doses of attenuated clone B.5 had a similar survival time.

Reassortants with an L RNA segment derived from the neurovirulent B1-1a virus (genotypes VAA, VAV, and VVA) were almost as neurovirulent as their virulent parent, and the mice died 6 to 9 days after infection. Reassortants with an L RNA segment derived from avirulent clone B.5 (AVA, AVV, and AAV) exhibited much lower virulence, but there was some variation among them. Reassortants with genotypes AVA and AVV were just as attenuated as the avirulent parent virus, B.5, in that they failed to kill mice at the lowest dilution tested (1:10) and had a computed ratio of over 10⁵ PFU/LD₅₀. Reassortants of genotype AAV showed slightly higher virulence, i.e., 10³ to 10⁵ PFU/LD₅₀, computed 14 days after infection. These reassortants produced some long-incubation-period deaths, between 15 and 21 days after injection, that were not seen with any other reassortants. Consequently, the AAV reassortants were 100- to 250-fold more virulent when mortality at 21 days (rather than 14 days) postinfection was used in the computation of LD₅₀ values.

These data indicated that the L RNA segment was the major determinant of the attenuation of avirulent clone B.5 but that clones bearing an L RNA segment derived from the attenuated parent varied in degree of attenuation. To confirm these differences, adult mice were injected i.c. with 700 PFU of both parental clones and of three selected reassortants, a virulent one, an avirulent one, and one of intermediate virulence, and the rate of viral replication in the brain was examined.

Figure 2 shows that the virulent parental clone, B1-1a (genotype VVV), replicated to high titer by day 3 and killed mice before day 7. The avirulent parental clone, B.5 (genotype AAA), barely replicated and did not kill any mice. The virulent reassortant, J18/6 (VAA), replicated to high titer by day 7 and killed 100% of mice between days 7 and 14. The avirulent reassortant, J18/33 (genotype AVA), barely repli-

FIG. 2. Brain replication, after i.c. injection of 700 PFU in adult mice, of parental and selected reassortant clones. A comparison is shown of a neurovirulent clone, B1-1a (genotype VUV), a nonneurovirulent clone, B.5 (genotype AAA), and three reassortants between clones B1-1a and B.5. The reassortants were J18/6 (genotype VAA, neurovirulent phenotype), J18/33 (genotype AVA, nonneurovirulent phenotype), and J26/19 (genotype AAV, intermediate phenotype).

cated and did not kill any mice. The reassortant of intermediate virulence, J26/19 (genotype AAV), replicated slowly and reached intermediate titers by day 14; it killed only 20% of mice, between days 15 and 21. These data confirm that the reassortant viruses with an L RNA segment derived from the avirulent parent exhibit different degrees of attenuation.

Temperature-sensitive phenotype. To map the temperature-sensitive phenotype of B.5 virus to the appropriate segment(s), reassortants were analyzed by a standard plaque assay on BHK-21 cells at both 33 and 39.8°C, and temperature sensitivity was expressed as the $\log_{10}$ (PFU at 33°C/PFU at 39.8°C). As shown in Table 2, virulent clone B1-1a is not temperature sensitive, and its $\log_{10}$ ratio is 0.0; conversely, the $\log_{10}$ ratio of avirulent clone B.5 is 5.3. Among the

reassortants, those with an L RNA segment from the virulent parent replicated well at 39.8°C ($\log_{10}$ ratios of 0.0 to 1.1), while those with an L RNA segment from the avirulent parent showed marked temperature sensitivity ($\log_{10}$ ratios of 3.8 to >6.2).

Plaque morphology and cellular host range. Avirulent clone B.5 exhibited a different plaque morphology than did virulent clone B1-1a and La Crosse and Tahyna viruses. In BHK-21 cells, clone B.5 produced smaller plaques than the virulent viruses, while in NA cells, clone B.5 produced a nonlytic focal plaque in contrast to the lytic plaques produced by the virulent viruses. When the reassortant clones were examined for plaque morphology (Table 2), it appeared that clones bearing an L RNA segment from the virulent parent produced large plaques in BHK-21 cells and that clones bearing an L RNA segment from the avirulent parent produced small plaques. Likewise, reassortant clones bearing an L RNA segment from the virulent parent replicated well and produced lytic plaques in NA cells, while reassortants bearing an L RNA segment from the avirulent parent replicated slowly in NA cells and produced focal plaques or no plaques at all.

We suggested (6) that NA cells might serve as a surrogate for the mouse brain in that neuroattenuated clone B.5 replicated very poorly in these cells. To determine the specificity of this restriction, clone B.5 and parent La Crosse virus were compared for their abilities to replicate in four selected mouse cell lines (C2C12 mouse myoblasts, NIH 3T3 fibroblasts, L929 fibroblasts, and P388D1 monocytoid cells). Figure 3 shows that clone B.5 replicated poorly or not at all in these cell types, in contrast to its vigorous replication in BHK-21 cells. Therefore, it appears that the restriction of clone B.5 is not specific for cells of neuronal origin.

DISCUSSION

Genetic determinants of neuroattenuation. The neuroattenuated clone B.5 is the first available California serogroup virus which is highly attenuated upon i.c. injection, with a lethality ratio over 10^6 -fold lower than those of wild-type strains or other viruses of the same genotype (Fig. 1). This phenotype is not merely an expression of the inability of

TABLE 2. Cell culture phenotypes of reassortants between a neuroattenuated clone (B.5) and a neurovirulent clone (B1-1a) of California serogroup viruses

Virus	Clone	Genotype	Virulence genotype	Plaque morphology in:		Sensitivity of NA cells ^b	Temperature sensitivity in BHK cells ^c
				BHK cells	NA cells ^a		
Parent	B.5	TLL	AAA	Small	Focal	6.2	5.3
	B1-1a	LTT	VVV	Large	Lytic	0.1	0.0
Reassortant	J18/6	LLL	VAA	Large	Lytic	0.4	0.7
	J18/8	LLT	VAV	Large	Lytic	0.3	0.8
	L20/1	LLT	VAV	Large	Lytic	0.1	1.1
	L20/7	LLT	VAV	Large	Lytic	0.5	0.5
	L20/8	LTL	VVA	Large	Lytic	1.0	0.0
	L20/12	LTL	VVA	Large	Lytic	0.5	0.0
	J18/33	TTL	AVA	Small	Focal	0.8	>6.2
	J18/34	TTL	AVA	Small	Focal	0.4	>3.3
	J26/44	TTT	AVV	Small	Focal	0.1	>4.3
	J18/81	TLT	AAV	Small	Focal	0.3	>4.3
	J18/38	TLT	AAV	Small	None	>7.0	>4.3
	J26/19	TLT	AAV	Small	None	>7.0	3.8

^a In NA cells, lytic plaques develop at 3 days, but focal plaques require 5 to 6 days.

^b Expressed as the $\log_{10}$ of the ratio PFU (BHK-21 cells)/PFU (NA cells).

^c Expressed as the $\log_{10}$ of the ratio PFU (33°C)/PFU (39.8°C).

FIG. 3. Replication of attenuated clone B.5 in selected cell cultures. The data show that B.5 replicates well in permissive BHK-21 cells at 37°C and that it replicates very poorly in mouse cell lines (C2C12 myoblasts, 3T3 fibroblasts, and NA neuroblastomas) at either 33 or 37°C. Data on NA cells are from reference 5.

clone B.5 to replicate, since it grows in BHK-21 cells to titers very similar to those of wild-type California serogroup viruses (6).

There appears to be a hierarchy of mouse cell restriction of attenuated clone B.5 in which suckling mouse brain is at the most permissive end of the spectrum. This phenomenon is also seen with standard viruses, such as wild-type La Crosse/original virus (15). These data on bunyaviruses are consistent with the longstanding observations that suckling mice are more permissive than older mice for a wide range of viruses (29, 33, 34). After i.c. injection, viruses tend to replicate more rapidly and to higher titer in young mice, and this phenomenon is evident within hours, i.e., long before immune induction has occurred. The mechanism of this age-specific differential permissiveness has yet to be explained (34).

The genetic studies reported here indicate that the L RNA segment is the major determinant of neurovirulence. This pattern contrasts sharply with the pattern of genetic determinants of neuroinvasiveness, since the M RNA segment, which encodes the viral glycoproteins, is primarily responsible for neuroinvasiveness (11, 16, 28). Our findings suggest that events other than virus attachment and entry may play a prominent role in the neurovirulence of California serogroup viruses.

Attenuation due to the L RNA segment implies that the polymerase of clone B.5 may function less effectively in certain cells. Thus, at permissive temperatures (33 to 37°C), attenuated clone B.5 has lost the ability to replicate in several mouse cell lines (Fig. 3) and in adult mouse brain, although it replicates well in BHK-21 cells and in the brains of suckling mice.

Polymerase genes have been found to be important determinants of virulence for certain other viruses. Type 1 herpes simplex virus shows greater neuroinvasiveness than does type 2 herpes simplex virus, due to its greater ability to spread along neural pathways from the eye to the CNS, and this difference was mapped to a viral DNA polymerase gene by using an intertypic recombinant (3). The ability of influenza virus to replicate in lung and cause viral pneumonitis is partially determined by polymerase genes (20, 29, 31). Cer-

tain strains of lymphocytic choriomeningitis virus are highly lethal in guinea pigs, with severe infection of lungs, lymph nodes, spleen, and adrenal glands, and lethality maps to the L RNA segment, which encodes the polymerase gene (24, 26). Viral transcription, replication, and morphogenesis are all possible stages which could be affected by an altered viral polymerase, and further work is required to elucidate which aspect of polymerase function is associated with attenuation.

Temperature sensitivity. The major genetic determinant of temperature sensitivity also appears to be the L RNA segment, since the six reassortants carrying an L RNA segment from the virulent parent exhibit minimal temperature sensitivity, while the six reassortants carrying an L RNA segment from the avirulent parent exhibit marked temperature sensitivity. This is consistent with our prior observation that temperature-sensitive revertants also showed a marked increase in mouse neurovirulence (6) and may offer an approach to fine mapping of a virulence determinant in the L RNA segment.

It is important to emphasize that the reduced neurovirulence of clone B.5, and of reassortants bearing an L RNA segment derived from clone B.5, is not caused by their temperature sensitivity. Replication of these virus clones is restricted in mice, at their body temperature of 37°C (35). In BHK-21 cells, clone B.5 replicates as well at 37 as at 33°C (Fig. 3) but is almost totally restricted at 39.8°C (6). Furthermore, clone B.5 appears to have lost its neurotropism in mosquitoes which are maintained at 22°C (1). Thus, neuroattenuation and temperature sensitivity appear to be independent phenotypic manifestations of a mutation(s) in the L RNA segment.

Polygenic effects. Although the L RNA segment appears to be the major determinant of neurovirulence, of plaque morphology, and of temperature sensitivity, the patterns of attenuation are somewhat irregular among the six reassortants bearing the L segment from avirulent clone B.5. Thus, these six reassortants vary in degree of avirulence, in temperature sensitivity, and in restriction in NA cells. These observations suggest that other RNA segments modulate the effect of the L RNA segment, particularly on an avirulent genetic background. When the L RNA segment is derived from the virulent parent, this modulation is much less apparent. This polygenic effect resembles the findings with the nonneuroinvasive Tahyna/181-57 virus (16), in which the M RNA segment was the dominant genetic determinant but the other two RNA segments modified the effect in reassortants bearing an M RNA segment derived from the attenuated parent.

In addition to its biologically altered L RNA segment, attenuated clone B.5 (genotype TLL) also has a mutation in its S RNA segment, since its N protein migrates more rapidly in PAGE than does the N protein of La Crosse/original virus (6). This mutation in the S RNA segment might contribute to the biological variability observed among reassortants with an attenuated L RNA segment.

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