Polygenic Control of Neuroinvasiveness in California Serogroup Bunyaviruses

CHRISTIAN GRIOT,¹ ANDREW PEKOSZ,^{1,2} DAVID LUKAC,^{1,2} STEVEN S. SCHERER,³
KARA STILLMOCK,¹ DAN SCHMEIDLER,¹ MICHAEL J. ENDRES,^{1†}
FRANCISCO GONZALEZ-SCARANO,^{1,3} AND NEAL NATHANSON^{1*}

*Department of Microbiology,¹ Molecular Biology Graduate Group,² and Department of Neurology,³
School of Medicine, University of Pennsylvania, 209 Johnson Pavilion, 36th and Hamilton Walk,
Philadelphia, Pennsylvania 19104-1001*

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The pathogenesis of the California serogroup bunyaviruses includes both extraneural and intraneural replicative phases that can be separated experimentally. The present study dissects the viral genetic determinants of extraneural replication. We have previously described two attenuated reassortant clones of California serogroup bunyaviruses which exhibit reduced neuroinvasiveness after subcutaneous inoculation into suckling mice. Clone B1-1a bears an attenuated middle RNA segment (neuroinvasiveness phenotype $\nu\alpha\nu$), and clone B.5 bears an attenuated large RNA segment (neuroinvasiveness phenotype $\alpha\nu\nu$). We prepared reassortant viruses between these two strains and found that the two attenuated gene segments acted independently and additively, since reassortants bearing two attenuated RNA segments were more attenuated than the parental clones. Reassortants bearing no attenuated RNA segments were much more neuroinvasive than either parental clone, indicating that a neuroinvasive strain can be derived from two attenuated clones. Pathogenesis studies demonstrated that after injection of 10^3 PFU, the attenuated reassortant clones did not replicate in peripheral tissue, failed to reach the brain, and did not cause disease. At a dose of 10^6 PFU, attenuated clones failed to replicate to a significant level in peripheral tissue and produced only a minimal passive plasma viremia during the first 24 h but nevertheless reached high titers in the brain and killed mice. Because of this result, we investigated the possibility that neuroinvasion occurs via retrograde axonal transport, by determining whether sciatic nerve sectioning could protect against virus infection after hind leg footpad inoculation. We found that nerve sectioning had no effect on lethality, ruling out this mode of entry and suggesting that passive viremia is likely to be sufficient for invasion of the central nervous system.

California serogroup bunyaviruses provide a powerful model to study the genetic determinants of virus virulence (3, 8, 10, 14–17, 20, 21, 24, 25, 36). La Crosse/original virus is the prototypic member of the California serogroup (1, 17) and is an important cause of pediatric encephalitis in the midwestern United States, where it produces over 300,000 clinically inapparent infections and approximately 75 reported cases of encephalitis annually (5, 19, 21, 22, 28, 39).

Experimental murine infection reproduces the clinical spectrum of illness seen in humans, from uniformly lethal to inapparent, depending on the virus strain, virus dose, route of infection, and age and possibly strain of the mice (15, 17, 21, 24, 26, 27). Subcutaneous virus inoculation simulates the natural route of transmission by the bite of infected *Aedes triseriatus* mosquitoes (1, 2, 17, 21) and permits the analysis of the sequential events that carry the virus from the site of entry to the central nervous system (21).

California serogroup bunyaviruses lend themselves to genetic analysis, since their genome is trisegmented and each segment encodes only a few viral proteins (for a review, see references 17 and 33). The large (L) RNA segment is translated into the L protein, the viral polymerase (7, 9). The middle (M) RNA segment encodes a single open reading frame that is translated into two structural glycoproteins, G1 and G2, and a nonstructural protein (NS_m) of unknown

function (3, 11, 23, 36). The small (S) RNA segment encodes the nucleocapsid protein (N) and another nonstructural protein (NS_s) of unknown function (4, 12, 13).

The pathogenesis of the California serogroup viruses includes two distinct phases: extraneural and intraneural replication (17, 21). For purposes of genetic analysis, we have called viruses that can penetrate the central nervous system after peripheral replication neuroinvasive and viruses that can replicate in cells within the central nervous system neurovirulent and assayed for each property by either subcutaneous or intracerebral inoculation, respectively (21). Using reassortant viruses and monoclonal antibody-resistant variants, we have determined that neuroinvasion and neurovirulence are not necessarily linked, since individual viruses can be both neuroinvasive and neurovirulent, be attenuated for both properties, or show a mixed virulence phenotype (8, 10, 14, 18, 24, 25).

In previous studies, we have described two reassortant clones, B1.1a and RFC 25/B.5 (referred to as B.5), both of which exhibited reduced neuroinvasiveness upon subcutaneous inoculation into suckling mice (8, 10, 25). Clone B1-1a (viral genotype LTT, neuroinvasiveness phenotype $\nu\alpha\nu$; the three-letter code designates the origin and neuroinvasiveness features of the L, M, and S RNA segments, respectively; see Materials and Methods) is a cross between neuroinvasive La Crosse/original virus and nonneuroinvasive Tahyna-181/57 virus and is attenuated because its M RNA segment was derived from the Tahyna-181/57 parent virus (25). Clone B.5 (TLL $\alpha\nu\nu$) has a mutation in the L RNA

* Corresponding author.

† Present address: Department of Medical Pathology, School of Medicine, University of California, Davis, CA 95616.

segment and, in addition to having a reduced neuroinvasiveness, is reduced in neurovirulence (8).

We constructed a panel of reassortants between these two clones in order to address the following points: (i) can a neuroinvasive reassortant clone be constructed from two attenuated parents, (ii) are the effects of attenuations in the two RNA segments additive, and (iii) are there differences in the sequential pathogenesis of the two attenuated clones, given that each one has a different attenuated RNA segment? Furthermore, the availability of a clone which does not replicate in peripheral tissue (but nevertheless invades the central nervous system) allowed us to ask whether California serogroup viruses invade the central nervous system directly from the blood or by moving along peripheral nerves via retrograde axonal transport, as has been shown for certain viremogenic clones of reovirus and poliovirus (31, 32, 35, 42).

MATERIALS AND METHODS

Animals. Outbred albino CD-1 Swiss mice (Charles River Laboratories, Wilmington, Mass.) were used in all experiments.

Reassortant viruses and cell culture. The construction, phenotyping, and genotyping of reassortants between the two parental virus clones B.5 and B1-1a is described elsewhere (10, 25). To indicate the viral genotype of such reassortant clones, we have used a three-letter code in which letters designate (in order of appearance) the origin of the L, M, and S RNA segments. RNA segments derived from La Crosse/original virus are designated L, while RNA segments derived from Tahyna-181/57 virus are designated T (3). For convenience in interpreting the results, in addition to its viral genotype, each parent and reassortant clone was assigned a neuroinvasiveness phenotype in which "v" represented a neuroinvasive RNA segment and "α" represented an attenuated RNA segment (20).

Clones B.5 (viral genotype TLL, neuroinvasiveness phenotype αvv) and B1-1a (LTT, vαv), both with markedly reduced neuroinvasiveness upon peripheral inoculation into suckling mice, were used as parental clones in this study. Clone B.5 is a variant virus derived by 25 passages of the reassortant clone RFC (also of genotype TLL) through BHK-21 cells, followed by triple-plaque purification of the passaged stock (10). Clone B1-1a is a reassortant clone which was derived from a cross between La Crosse/original virus and Tahyna-181/57 virus (25).

BHK-21 cells (clone 13; American Type Culture Collection, Rockville, Md.) were routinely maintained in minimum essential medium (JRH Biosciences, Lenexa, Kans.) supplemented with 5% heat-inactivated fetal bovine serum (GIBCO Life Technologies, Grand Island, N.Y.) and 1% L-glutamine (JRH Biosciences). The virus titer of reassortant working stocks was determined by the standard plaque assay on BHK-21 cells and expressed as PFU per milliliter (24).

Determination of neuroinvasiveness of reassortant clones. Suckling mice (3 to 4 days old) were injected subcutaneously at two different locations: the nuchal region (50 μl) or the right hind leg footpad (10 μl). Five to ten mice were used per 10-fold virus dilution each in minimum essential medium. Mortality was recorded daily for up to 14 days, and the 50% lethal doses (LD₅₀) were computed by the method of Reed and Muench (34). Each inoculated reassortant clone was titrated simultaneously in BHK-21 cells as described above, and the ratio of the two titers (PFU/LD₅₀) constitutes the neuroinvasiveness index (NI) (10).

Indirect immunofluorescence labeling. Animals were sacrificed when moribund. Tissue blocks (thorax and abdomen sections) were frozen at -80°C, sectioned in a cryostat at 6 μm, air dried, fixed in 4% paraformaldehyde (Sigma, St. Louis, Mo.) for 10 min at room temperature, and washed three times with phosphate-buffered saline (PBS; pH 7.4; GIBCO) containing 1% Nonidet P-40 (Sigma). Sections were preincubated with a blocking solution of 4% bovine serum albumin (BSA; Sigma) in PBS for 30 min at room temperature. A hyperimmune rabbit anti-La Crosse/original virus antiserum (1:50; diluted in PBS containing 4% BSA) was employed in the first step and incubated for 2 h at 37°C, and this was followed by a fluorescein-5-isothiocyanate-conjugated goat anti-rabbit immunoglobulin G F(ab')₂ fragment (Organon Teknica, Durham, N.C.) diluted to 1:100 in PBS containing 4% BSA and 0.05% Evans blue (Sigma) as a counterstain (24). The sections were examined for viral antigen by epifluorescence microscopy.

Tissue titration after injection of virus into the nuchal region or footpad. A standard protocol was used to monitor the replication and pathogenesis of the selected reassortant clones (8, 10, 24). Infected mice were euthanized at predetermined intervals, and tissue samples (blood, hind leg thigh muscle, and brain) from three animals were pooled and prepared as a 10% suspension in minimum essential medium with 2% fetal bovine serum (24).

To monitor virus replication after footpad inoculation, a variant protocol was used in which 200 PFU of clone B1-1a (LTT) was injected subcutaneously into the right hind limb footpad and tissue samples (left and right hind limb footpads, left and right thigh muscles, superior and inferior spinal cord, brain, and blood) of five animals were harvested and pooled as described above. The spinal cord was removed by microdissection; the superior spinal cord blocks included the cervicothoracic cord segments, while the inferior spinal cord blocks included the lumbosacral cord segments (42).

The virus titers in all of the tissue homogenates and in the plasma were determined by standard plaque assay on BHK-21 cells. The titers were recorded as PFU per milligram of tissue or PFU per microliter of plasma, with a sensitivity of 1 PFU/2 mg or 1 PFU/20 μl (24).

Sciatic nerve transection. Ten 4-day-old suckling mice were anesthetized (Methoxyflurane; Pitman-Moore, Mundelein, Ill.). The right sciatic nerve was transected with iridectomy scissors with the aid of a dissecting microscope. Control animals underwent sham operations by exposing but not transecting the sciatic nerve. The adequacy of the nerve section was demonstrated by postoperative findings of hind limb analgesia and paresis (42). Clone B1-1a was inoculated into the hind limb footpad 24 h after nerve sectioning, mortality and survival time were recorded, and the NI was calculated as described above.

RESULTS

Neuroinvasiveness of reassortant clones. We had previously identified two clones with reduced neuroinvasiveness after subcutaneous inoculation, B1-1a and B.5 (8, 10, 25). Clone B.5 (viral genotype TLL, neuroinvasiveness phenotype αvv; see Materials and Methods) is a reassortant between La Crosse/original virus and Tahyna-181/57 virus that had been modified by 25 serial passages in BHK-21 cells. Its L RNA segment is known to be responsible for reduced neurovirulence in adult mice (8). Clone B1-1a is also a reassortant between La Crosse/original virus and Tahyna-181/57 virus and has the viral genotype (LTT) reciprocal to that of clone

TABLE 1. Characterization of reassortant clones between the two parental clones B.5 and B1-1a

Clone	Genotype ^a	Phenotype ^b	Log ₁₀ PFU/ml ^c	Log ₁₀ LD ₅₀ /ml ^d	NI (log ₁₀ PFU/LD ₅₀)
B.5	TLL	$\alpha\nu\nu$	7.4	2.6 ^e	4.8 ^e
J26/19	TLT	$\alpha\nu\nu$	8.3	4.8 ^e	3.5 ^e
J18/33	TTL	$\alpha\alpha\nu$	7.0	1.7	5.3
J26/44	TTT	$\alpha\alpha\nu$	7.0	1.5 ^e	5.5 ^e
J18/6	LLL	$\nu\nu\nu$	9.5	8.3	1.2
L20/1	LLT	$\nu\nu\nu$	8.4	9.3 ^e	-0.9 ^e
L20/8	LTL	$\nu\alpha\nu$	8.2	5.8	2.4
B1-1a	LTT	$\nu\alpha\nu$	7.3	4.4 ^e	2.9 ^e

^a L, La Crosse/original virus-derived RNA segment; T, Tahyna-181/57 virus-derived RNA segment (see Materials and Methods).

^b α , attenuated (nonneuroinvasive allele); ν , neuroinvasive allele (see Materials and Methods).

^c Titration of BHK-21 cells.

^d Titration in suckling mice injected subcutaneously in the nuchal region.

^e Mean from two determinations.

B.5 (25). Like other reassortants with an M RNA segment derived from Tahyna-181/57 virus, clone B1-1a (neuroinvasiveness phenotype $\alpha\nu\nu$) has markedly reduced neuroinvasiveness (25). A panel of reassortants, representing all six possible genotypes, was constructed between clones B.5 and B1-1a. All the progeny clones (including the parental clones) were then titrated for neuroinvasiveness by subcutaneous inoculation into suckling mice.

Table 1 shows the NI for the eight clones. There are several important, interrelated findings. First, the reduced neuroinvasiveness of clone B.5 ($\alpha\nu\nu$) was clearly associated with the L RNA segment, since reassortant clone J18/6 ($\nu\nu\nu$), in which the L RNA segment of clone B.5 was replaced with the L RNA segment of clone B1-1a, was neuroinvasive, consistent with our previous findings with La Crosse/original virus (25). Second, the effect of attenuated RNA segments (clones with neuroinvasiveness phenotype $\alpha\alpha\nu$) appeared to be independent and additive. Clones with the two attenuated RNA segments (J18/33 and J26/44) were 10- to 100-fold more attenuated than were clones with a single attenuated RNA segment. On the other hand, clones lacking either attenuated RNA segment ($\nu\nu\nu$) were at least 100- to 1,000-fold more neuroinvasive than clones with a single attenuated RNA segment.

The viral genotype also had some influence on survival time, since animals injected with clones bearing both neuroinvasive L and M RNA segments died between 4 and 6 days later while animals injected with the other clones had a longer survival time, ranging from 6 to 9 days (data not shown). All of the clones replicated well in BHK-21 cells, but stocks of different clones varied in titer from $10^{7.0}$ to $10^{9.5}$ PFU/ml. Clones with a neuroinvasiveness phenotype of $\nu\nu\nu$ produced the highest titers.

Pathogenesis of reassortant clones. We then compared the sequential replication of clones representing each viral phenotype following the subcutaneous inoculation of 10^3 PFU per animal. Figure 1 (lower panel) shows that clone L20/1 ($\nu\nu\nu$) appeared in blood, striated muscle, and brain 24 h after inoculation. The titer in the brain rose rapidly, and all animals died within 3 days. None of the other three clones replicated in either muscle or brain, nor did they produce a plasma viremia. As the dose of 10^3 PFU was below the LD₅₀, there was no mortality (Fig. 1, upper panel).

Since the panel of reassortants had attenuating mutations in two different RNA segments, it was of interest to determine whether these viruses exhibited qualitative differences in their pathogenesis. Four clones, representing each of the neuroinvasiveness patterns shown in Table 1, were injected subcutaneously into suckling mice at a high dose (10^6 PFU per animal) which would produce 100% mortality. Figure 2 shows that clone L20/1 ($\nu\nu\nu$) replicated to a high titer in striated muscle, blood, and brain and killed animals within 2 days. The three attenuated clones, B1-1a ($\nu\alpha\nu$), B.5 ($\alpha\nu\nu$), and J26/44 ($\alpha\alpha\nu$), appeared in the brain within 24 h, replicated to a high titer, and killed mice in 4 days (Fig. 2).

To further investigate the apparent differences in the ability of the attenuated reassortant clones to replicate in peripheral tissues, we examined selected tissues for the presence of viral antigen. Suckling mice (two to three animals for each reassortant) were injected subcutaneously with 100 LD₅₀ of each clone, the mice were euthanized when moribund, and their tissue sections were examined for the expression of viral antigens. Table 2 shows a clear hierarchy

FIG. 1. Replication of the two parental reassortant clones, B.5 and B1-1a, and selected reassortants after subcutaneous injection of 10^3 PFU into suckling mice. Shown is a comparison of the neuroinvasive clone L20/1 and the attenuated clones B.5, J26/44, and B1-1a. Clone L20/1 replicated in striated muscle, blood, and brain and killed animals after 3 days, while the other clones failed to replicate or kill mice.

FIG. 2. Replication of reassortant clones after subcutaneous injection of 10^6 PFU into suckling mice. Clone L20/1 replicated to a high titer in striated muscle, blood, and brain and killed animals after 2 days. Clones B.5, J26/44, and B1-1a produced an initial plasma viremia, appeared in the brain after 24 h, and killed animals after 4 days.

in the level of virus replication in peripheral tissue. Clone L20/1 ($\nu\nu\nu$) replicated widely in striated muscle and in neurons of the parasympathetic plexus in the intestine. Clones B1-1a ($\nu\alpha\nu$) and B.5 ($\alpha\nu\nu$) were detected only at minimal levels in striated muscle. Clone J26/44 ($\alpha\alpha\nu$) was not detected in any tissue.

Since the attenuated clones did not replicate in peripheral tissue, it was unclear how these clones reached the brain so rapidly. For instance, in the initial assays, clone B1-1a ($\nu\alpha\nu$) failed to show virus in muscle or blood at 24 or 48 h. We then examined the animals at 3, 6, and 12 h postinjection and found that following the high-titer inoculation, the attenuated clones produced a passive plasma viremia, peaking at 6 to 12 h after infection and disappearing again by 24 h. After the infection of the central nervous system was well established, all of the attenuated clones showed a minimal plasma viremia which was not relevant to viral invasion of the central nervous system.

Pathway of neural spread of reassortant clone B1-1a. Axonal spread is an important mechanism for viral penetration

of the central nervous system (31, 32, 41, 42). We surmised that if neural spread were to occur in the California serogroup bunyaviruses, a neuro-adapted clone, like clone B1-1a that could not replicate in striated muscle but nevertheless invades the brain, might enter the central nervous system via the neural route. Therefore, we investigated the possibility that clone B1-1a reaches the central nervous system by retrograde axonal transport along peripheral nerves. First, clone B1-1a was titrated by using two different subcutaneous injection sites: the nuchal region (the site which was primarily used throughout this study), and the footpad of the hind leg. There was a consistent difference between the two routes, since after footpad injection, the NI was 20 PFU/LD₅₀, with a median survival time of 3.6 days, compared with an NI of 200 PFU/LD₅₀ and median survival time of 6.5 days after injection of clone B1-1a into the nuchal region (Table 3).

We reasoned that clone B1-1a inoculated into the footpad might reach peripheral nerve endings more rapidly than virus injected into the nuchal region and therefore might be

TABLE 2. Distribution of viral antigen in peripheral tissue of suckling mice moribund after subcutaneous inoculation of selected reassortant clones

Clone	Genotype	Phenotype	Log ₁₀ PFU/animal ^a	Viral antigen localization ^b	
				Striated muscle	Gut plexus
L20/1	LLT	vvv	3	++	+
B1-1a	LTT	vav	6	±	—
B.5	TLL	avv	6	±	—
J26/44	TTT	avv	6	—	—

^a Virus inocula were chosen to produce 100% mortality in suckling mice.

^b ++, sections from all animals showed intense viral antigen staining throughout the tissue; +, sections from all animals showed viral antigen staining, but only in single cells; ±, minimal antigen staining in some but not in all sections examined; —, no viral antigen detected in any of the sections. Each entry is based on two to three sections from each of two to three mice.

spreading to the central nervous system by a neural pathway. An experiment was conducted, using 200 PFU per animal, to determine whether sectioning of the sciatic nerve, which interrupts axonal transport, would prevent spread of virus to the central nervous system after footpad injection. The results are shown in Table 3 and clearly indicate that the sciatic nerve section afforded no protection from central nervous system invasion. In all three groups (animals with nerve transection or sham operation and animals with no surgery at all) median survival time was in the range of 3.6 to 4 days and mortality was over 80%.

Since this result was unexpected, an additional experiment was conducted, again using 200 PFU of clone B1-1a per animal, injected subcutaneously into the footpad, to ascertain whether virus would appear at different times in the inferior and superior spinal cord and in the brain. Such a gradient has been described in reovirus infection where central nervous system penetration occurs through the sciatic track (31, 32, 42). Figure 3 shows that virus replicated first in the injected hind limb footpad beginning at 12 h, then initiated a modest plasma viremia which was first detected at 36 h, and then rapidly replicated in the brain. Most importantly, there was no evidence that the virus invaded the inferior spinal cord before spreading to the superior cord and brain; rather the pattern was what would be expected after viremic spread to the central nervous system.

DISCUSSION

Polygenic effects on neuroinvasiveness. We have constructed reassortants between two clones, B1-1a and B.5,

TABLE 3. Failure of sciatic nerve transection to alter mortality or survival time of suckling mice injected with 200 PFU of clone B1-1a (vav) into the hind leg footpad

Site of virus injection	Sciatic nerve ^a	Survival time (days)		Mortality (%) ^b
		Median	Range	
Footpad	Cut	3.6	3-4	87.5
	Sham	3.6	3-4	87.5
	Uncut	4.1	3-5	81.8
Nuchal region	Uncut	6.5	6-7	43.3

^a The sciatic nerve was transected as described in Materials and Methods. Uncut, no surgery at all was performed; sham, animals underwent sham operation.

^b *n* = 10.

both of which exhibit reduced neuroinvasiveness upon peripheral inoculation into suckling mice (10, 25). The attenuation of these clones has been mapped to two different RNA segments: clone B1-1a bears an attenuated M RNA segment, which codes for the two glycoproteins G1 and G2 as well as NS_m, and clone B.5 bears an attenuated L RNA segment, which codes for the viral polymerase. Upon peripheral inoculation of these reassortants into suckling mice, we noted that the two segments acted additively and independently, so that progeny clones lacking either attenuated L or M RNA segments were as neuroinvasive as prototypic La Crosse/original virus and reassortants with both attenuated L and M RNA segments exhibited greater attenuation than either of the attenuated parental clones.

It has already been reported that reduced neuroinvasiveness of attenuated clones of California serogroup viruses mapped to the M RNA segment (25). Since the M RNA segment codes for the viral surface glycoproteins G1 and G2, it can be reasoned that events like virus attachment to and/or entry into cells play an important role in the neuroinvasiveness of California serogroup bunyaviruses (25, 41). The present study shows that in addition, the viral polymerase, the product of the L RNA segment, can contribute to an attenuated biological phenotype. Attenuation due to the L RNA segment implies that the polymerase of clone B.5 (and reassortants bearing the B.5-derived L RNA segment) may function less effectively in cells of certain peripheral tissues. This is supported by the finding that only a minimal amount of viral antigen was detectable in striated muscle after inoculation of clone B.5.

There are several reports which show that viral polymerase genes can be important determinants of the virulence of other viruses (6, 29, 37, 38). For example, herpes simplex virus type 1 is more neuroinvasive than type 2, since it can spread along neural pathways to the central nervous system; this difference has been mapped to the viral DNA polymerase gene (6). Influenza virus replication in lung tissue is also partially determined by polymerase genes (29, 37, 38).

Pathogenesis of reassortant clones. Studies of the sequential spread of virus after subcutaneous inoculation of 10³ PFU into suckling mice indicated that reassortants with no attenuated RNA segments replicated to a high titer in striated muscle, blood, and brain, killing all the animals, while clones with an attenuation in the L and/or M RNA segment failed to replicate and did not kill any animals.

At the large dose of 10⁶ PFU per animal, even the most attenuated reassortants invaded the brain and produced a lethal encephalitis. Since these attenuated clones replicated very poorly in peripheral tissues, it was unclear how they were transported to the central nervous system, where they appeared within 24 h after subcutaneous injection. We considered two possibilities: passive viremia and neural spread. Reexamination of the first 24 h after injection revealed that all of the viruses produced a plasma viremia beginning at 3 to 6 h, peaking at 12 h, and dropping or disappearing at 24 h. We calculated that the diffusion of <5% of the injected inoculum into the circulation (lymphatics and/or blood) would be sufficient to produce the observed level of passive plasma viremia.

We also considered the possibility that the attenuated clone B1-1a utilizes the neural route to the central nervous system, since its parent Tahyna-181/57 virus had been derived by neuro-adaptation in mice (30). Titration of clone B1-1a by two different subcutaneous sites of injection showed that the virus was 10 to 100 times more lethal and killed animals 2 to 3 days earlier after hind leg footpad

FIG. 3. Replication of reassortant clone B1-1a (vav) after subcutaneous injection of 200 PFU into the right hind leg footpad of suckling mice. Virus invasion and replication in the central nervous system occur simultaneously in the brain and superior spinal cord and slightly later in the inferior spinal cord.

inoculation than after nuchal region inoculation. Since the accessibility of the virus to peripheral nerve endings in the footpad might be greater than in the nuchal region (40), we surmised that clone B1-1a might be spreading by the neural route. However, to our surprise, sciatic nerve transection failed to protect the animals. A final experiment confirmed that after hind leg footpad inoculation, virus appeared in the brain before it appeared in the inferior spinal cord, which is consistent with viremic invasion of the central nervous system (31, 41, 42). The exact mechanism by which California serogroup viruses cross the blood-brain barrier remains unclear and is currently under investigation.

We had speculated that clones with attenuating mutations in different RNA segments might exhibit different patterns of pathogenesis when injected in doses large enough to be lethal. However, our observations failed to demonstrate a major difference in pathogenesis, consistent with the conclusion that passive viremia was the most likely mechanism for invasion of the central nervous system by all our attenuated virus clones.

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