

## Comparative Neurovirulence of Selected Vesicular Stomatitis Virus Temperature-Sensitive Mutants of Complementation Groups II and III

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Weanling mice were inoculated intracerebrally with selected vesicular stomatitis virus (VSV) complementation group II and III temperature-sensitive (*ts*) mutants. Of the VSV *ts* mutants studied, only *ts* G32, a group III complementation mutant, appeared neurovirulent. Interestingly, neither the capacity to replicate in central nervous system tissue nor the ability to replicate in certain neurally derived continuous cell lines at semipermissive or nonpermissive temperatures appeared different among the VSV *ts* mutants employed. Finally, the pathological alterations in central nervous system tissue produced by VSV *ts* G32 were entirely different than those produced by G31 VSV *ts* in the group III mutant. These studies support the hypothesis that both the virological and neuropathological features produced by different VSV *ts* mutants are dependent upon the unique characteristics of each mutant, rather than upon a common biochemical defect shared by all members of a complementation group.

Temperature-sensitive (*ts*) mutants of a large number of viruses have been studied to delineate biochemical, morphological, replicative and genetic events underlying host-virus interactions. We, along with other groups, have used *ts* mutants in vivo to investigate the pathogenesis of viral infection (1-20). Among the more important studies preceding ours, those involving *ts* mutants of reovirus in rats (6, 17) and *ts* mutants of measles virus in hamsters (8) are especially noteworthy for providing insights into the capacity of *ts* mutants to alter the pathogenesis of viral infection.

In previous publications (1, 3-5, 15, 16) our group reported on a number of animal model systems employing wild-type (wt) vesicular stomatitis virus (VSV) as well as several VSV *ts* mutants. In particular, comparisons were made between the neurovirulence of wt VSV and certain VSV *ts* mutants in complementation groups I through IV. For example, whereas intracerebral inoculation of wt VSV invariably resulted in the rapid death of mice unaccompanied by substantial neuropathological findings, certain *ts* mutants were associated with a markedly attenuated infectious process and strikingly varied neuropathological findings. Thus, infection produced by VSV *ts* G31 (complementation group III) resulted in an extensive spongiform encephalopathy (1, 15), whereas VSV *ts* G41 (complementation group IV) produced a subacute infection without spongiform changes but associated with primary demyelination (4, 16). Both pat-

terns of disease were different from each other as well as from wt virus infection.

Several questions were raised by these studies. First, was a similar neurovirulence capacity expressed by all members of a complementation group or was neurovirulence the unique property of an individual *ts* mutant within a complementation group? Second, were there neurovirulence characteristics common to various *ts* mutants which explained the host-virus central nervous system (CNS) interactions observed with VSV *ts* mutants?

To examine these questions, we infected mice with several different complementation group II and III VSV mutants and compared CNS changes in such mice with those previously studied in animals infected with either *ts* mutants G22, G31, or G41. Results of these studies suggest that virus-induced alterations in the CNS of mice infected with VSV *ts* mutants are dependent on the biological characteristics of each *ts* mutant and not on a biochemical alteration common to all *ts* mutants of a given complementation group. In addition, neither the ability of VSV *ts* mutants to grow in a variety of neurally derived cell lines in vitro at permissive or semipermissive temperatures nor replication in vivo permits one to predict the neurovirulent potential of the VSV *ts* mutants.

### MATERIALS AND METHODS

**Animals.** Outbred Swiss mice, 3 to 4 weeks old, were purchased from Scientific Products, Arlington

Heights, Ill. All mice were housed in groups of 5 to 10 in polycarbonate cages and were provided with food and water ad libitum. Ambient room temperature was  $21^{\circ}\text{C} \pm 1^{\circ}\text{C}$ .

**Viruses.** The *ts* mutants G21, 052, 023, and G32 were generously provided by Robert Wagner (University of Virginia, Charlottesville, Va.). These *ts* mutants have previously been described by Wagner and others (13, 20). Each of these mutants was repeatedly plaque purified in Wagner's laboratory as well in as our own, and stocks were prepared by low-multiplicity infection of BHK-21 cells as previously described (15). Yields of *ts* mutants, as determined by plaquing in BHK-21 cells at  $31^{\circ}\text{C}$ , were  $1.0 \times 10^9$  plaque-forming units (PFU) per ml for *ts* G21,  $3.2 \times 10^8$  PFU per ml for *ts* 052,  $4.1 \times 10^8$  PFU per ml for *ts* 023, and  $1.5 \times 10^9$  PFU per ml for *ts* G32. Yields of plaque-purified *ts* mutants G31, G22, and G41 were as previously described (15).

**Virus infection.** Mice were inoculated with approximately  $10^7$  PFU of each of the VSV *ts* mutants, i.e., by injecting 0.03 ml of inoculum through a 25-gauge needle inserted while animals were maintained under light anesthesia. Preliminary experiments had indicated that with the exception of *ts* G32, all other mutants were avirulent at lower doses. All suspensions of virus were diluted in Hanks balanced salt solution. In contrast to results obtained with the intracerebral route of injection, administration of VSV *ts* mutants intraperitoneally did not result in clinical illness or death.

**Cell lines used for cultures.** BHK-21 cells were originally obtained from International Scientific Industries (Cary, Ill.) and grown to confluence in minimal essential medium with Earle salts supplemented with 7% fetal calf serum (virus-screened; GIBCO Laboratories, Grand Island, N.Y.), and other supplements as described before (15).

Murine neuroblastoma cells (NB) N2a were propagated as monolayer cultures in Dulbecco modified minimal essential medium supplemented with 10% fetal calf serum 100 U of penicillin, and 20  $\mu\text{g}$  of gentamicin. Rat C-6 glioma cells were originally obtained from M. Christensen, Northwestern University and grown in the same medium as NB cells.

For determination of the growth capacity of the VSV *ts* mutants in various cell lines, BHK-21, NB, and glioma cells were employed according to the following protocol. Cells at a density of  $2 \times 10^5$  cells per ml were infected with a *ts* mutant at a multiplicity of infection of from 1 to 10. Virus adsorption to the cells proceeded for 15 min at room temperature ( $24^{\circ}\text{C}$ ). Sealed replicate tubes with medium were then distributed to water baths for culture at 31, 37, and  $39^{\circ}\text{C}$ . The water baths were maintained at their respective temperatures throughout the culture period with a variation of temperature of  $\pm 0.01^{\circ}\text{C}$ . Replicate cultures at the various temperatures were obtained at zero time as well as at 4, 8 and 12 h after infection.

**Plaque assay.** BHK-21 cells were cultured in six-well plates (35 by 10 mm, FB-6TC; Linbro Co., New Haven, Conn.) in 2 ml of BHK-21 growth medium as previously described (15). Plaque assays were performed with a conventional agar overlay containing neutral red as described before (15).

**Preparation of organs.** Organs were obtained after exsanguination and killing, prepared as a 10%

(wt/vol) suspension in Hank balanced salt solution and stored frozen at  $-70^{\circ}\text{C}$  for subsequent titration.

**Pathology studies.** Two to three animals were sacrificed at 1- to 2-day intervals from 4 to 19 days postinfection with VSV *ts* G32. No detailed pathological studies were performed with the other VSV *ts* mutants because of the absence of substantial neuro-pathogenicity associated with these mutants. Mice were sacrificed by total body perfusion with chilled 3% glutaraldehyde in phosphate buffer (pH 7.4) under general anesthesia. Brain and spinal cords were removed, and sections approximately 1-mm-thick were postfixed in osmic acid, dehydrated through a graded series of ethyl alcohol, cleared in propylene oxide, and embedded in Epon; 1- $\mu\text{m}$ -thick sections were stained with toluidine blue and examined under light microscopy.

## RESULTS

The majority of mice inoculated intracerebrally with *ts* mutants G21, 052, and 023 remained alive even after receiving large amounts of virus (Table 1). In contrast, mice inoculated with *ts* G32 developed definite neurological disease with characteristic ruffled fur, lethargy, and hind-limb paralysis (Table 1); about 40% of the mice succumbed to infection 7 to 19 days after virus inoculation, whereas 20% survived but with persistence of hind-limb paralysis. *ts* G32 VSV, although neurovirulent, was less virulent than either VSV *ts* mutant G22 or G31 (16).

Mice were next infected with the respective VSV *ts* mutants, and viral recovery of the CNS as well as of an extraneural site were determined (Table 2). Brain, spinal cord, and kidney viral titers were indistinguishable between *ts* G21, a weakly neurovirulent *ts* mutant, and *ts* G32, a more neurovirulent mutant (Table 2). The major virological difference in the data was that, after viral inoculation, *ts* G32 VSV was recovered from brains of infected mice for 5 days whereas *ts* G21 infection permitted recovery of virus for only 3 days. *ts* 023 behaved in a similar manner to *ts* G21 in terms of viral recovery from infected mice (Table 2). Interestingly, no virus was isolated from neural or extraneural sites after *ts*

TABLE 1. Mortality for 3- to 4-week-old Swiss mice after intracerebral inoculation with various VSV *ts* mutants

Inoculation (PFU) (no. of mice)	VSV <i>ts</i> Mutant	Days postinfection of individual mouse death <sup>a</sup>
$10^7$ (10)	<i>ts</i> G21	6, 7 (8 alive)
$10^7$ (10)	<i>ts</i> 052	6, 6 (8 alive)
$10^7$ (10)	<i>ts</i> 023	8 (9 alive)
$10^7$ (20)	<i>ts</i> G32	7, 8, 9, 10, 18, 19 7, 9, 10, 11 (4 with hind-limb paralysis and 6 alive)

<sup>a</sup> The number of mice surviving and their condition is given within parentheses.

TABLE 2. Recovery of various *ts* VSV mutants from 3- to 4-week-old Swiss mice after intracerebral inoculation with  $10^7$  PFU of VSV *ts* mutants

Complementation group	VSV <i>ts</i> Mutant	Brain <sup>a</sup>			Spinal Cord <sup>a</sup>			Kidney <sup>a</sup>		
		Titer	Day <sup>b</sup>	Duration <sup>c</sup>	Titer	Day	Duration	Titer	Day	Duration
II	<i>ts</i> G21	$5 \times 10^4 \pm 0.5$	3	3	$1 \times 10^2 \pm 0.4$	3	3	$1 \times 10^2 \pm 0.6$	3	3
	<i>ts</i> 052	— <sup>d</sup>	—	—	—	—	—	—	—	—
III	<i>ts</i> 023	$5 \times 10^3 \pm 0.2$	3	3	$2 \times 10^2 \pm 0.3$	3	3	ND <sup>e</sup>	—	—
	<i>ts</i> G32	$3 \times 10^4 \pm 0.3$	3	5	$3 \times 10^2 \pm 0.4$	3	3	$2.5 \times 10^2 \pm 0.2$	3	3

<sup>a</sup> Values represent peak arithmetic mean of three individual organ titrations  $\pm 1$  standard error of the mean.

<sup>b</sup> The day of which peak organ titer was achieved.

<sup>c</sup> Values represent the number of days virus was recovered from specified organ.

<sup>d</sup> —, No detectable virus.

<sup>e</sup> ND, Not determined.

052 inoculation (Table 2). Viral isolates, recovered from the brains and spinal cords of mice infected with each of the VSV *ts* mutants, were temperature sensitive and, hence, not revertant virus (data not shown).

Since previous studies had shown a marked predilection for VSV replication in neurons of the CNS (1), we next considered the possibility that the neurovirulence of VSV *ts* G32 might be a function of its enhanced replicative ability in neural cells. To test this hypothesis, NB, glioma, and BHK-21 cells (a standard assay cell line) were employed in a growth-curve analysis at various temperatures. Figures 1A through D depict the results when *ts* mutants G21, 023, G32, and 052 were culture grown in this manner. It is apparent that all mutants grew optimally, as expected, at 31°C. NB cells appear to replicate all VSV *ts* mutants best among the cell lines used here (Fig. 1A through D). Nevertheless, in studying either the magnitude or kinetics of replication in the growth assays, one is unable to differentiate neurovirulent *ts* G32 from the least virulent mutant *ts* 023 at any temperature of incubation. Thus, all VSV *ts* mutants replicate approximately the same at 31 and 37°C in all the cell lines used in this study.

Finally, mice infected with *ts* G32 were studied sequentially for histopathological lesions. By 8 days after infection, the spinal cords of mice could be shown to be infiltrated by large numbers of mononuclear cells. By 19 days postinfection, the spinal cords of infected mice showed numerous perivascular cuffs, composed of mononuclear cells, as well as a general decline in the number of neurons. By 10 to 12 days postinfection, discrete foci of primary demyelination were also apparent in the white matter of the spinal cord (5). Again, as demonstrated in previous publications, these histopathological alterations are completely different than those seen after VSV *ts* G31 infection.

## DISCUSSION

Infection of Swiss mice with VSV *ts* G32 produced an inflammatory disease of the CNS characterized by an acute to subacute course with hind-limb paralysis and both gray and white matter alterations. Infected mice succumbed 10 to 12 days after infection, and 10 to 20% survived and remained paralyzed. Overall, 60% of the infected mice developed neurological disease. In contrast, other complementation group II and III VSV *ts* mutants were practically avirulent when injected intracerebrally into mice.

It is interesting to contrast *ts* G32 infection with both wt VSV as well as other VSV *ts* mutants. Table 3 provides a summary of comparative clinical and virological features of a number of VSV *ts* mutants. It is important to note that the data derived for a number of the mutants was obtained previously. Nevertheless, even though the observations were made at different times, our experience has been that the features of these diseases are consistently present. Infection of mice by wt VSV was invariably fatal in 2 to 3 days and resulted in pathological changes consisting of ependymal necrosis and viral cytoplasmic inclusions in anterior horn neurons (1, 15). Viral titers in the brains and spinal cords of infected mice reached a peak of  $10^7$  to  $10^8$  PFU per ml (Table 3). *ts* G31 produced a slower CNS infection characterized by hind-limb paralysis and death 6 to 9 days after inoculation. Status spongiosus, confined to the gray matter of the spinal cord, was the main pathological feature of this infection (1, 15). Viral titers in the brain and spinal cord of mice infected with *ts* G31 were only 1% of those seen after VSV wt infection (15) (Table 3).

Infection of BALB/c mice with VSV *ts* G41 (complementation group IV) produced the most chronic course of all of the mutants we have tested (16). Of the infected animals, 60% devel-

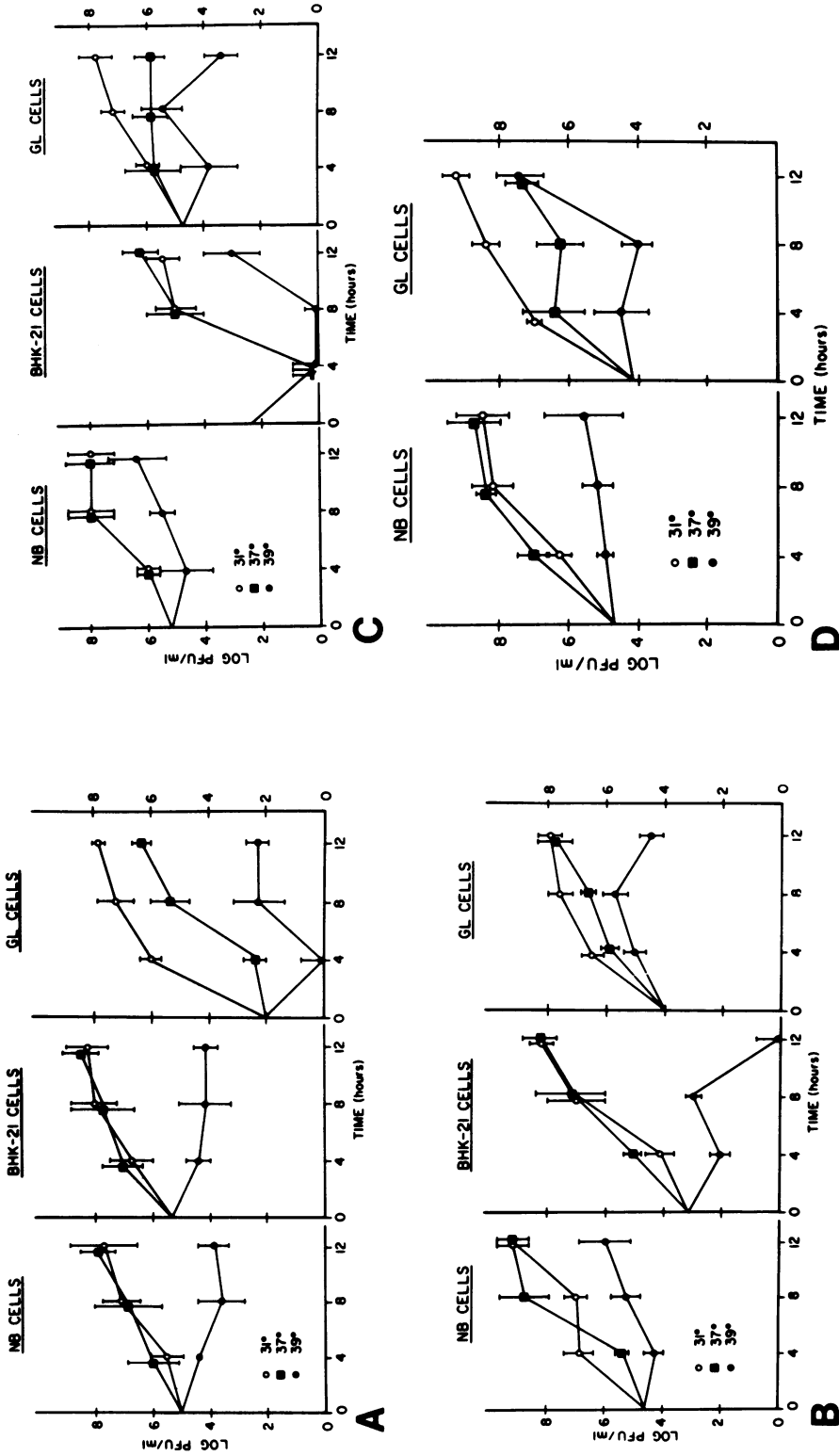


FIG. 1. Growth of VSV *ts* mutants 023 (A), G32 (B), G21 (C), and 052 (D) in NB, BHK-21, and rat C-6 glioma cells. Cells were infected at a multiplicity of infection of 1 to 10. Virus adsorption occurred for 15 min at 24°C. Sealed replicate cultures of infected cells at a density of  $2 \times 10^6$  cells per ml were distributed to water baths for culture at 31, 37, and 39°C. Samples were harvested at time zero as well as at 4-h intervals, and those containing *ts* mutant virus were stored at -70°C and subsequently assayed on BHK-21 cell monolayers at 31°C. Yields of virus were plotted as  $\log_{10}$  PFU per ml  $\pm$  1 standard error.

TABLE 3. Clinical and virological characteristics of intracerebral infection with different VSV *ts* mutants and wt VSV

VSV <i>ts</i> mutant	Complementation group	Peak virus titer from brain, spinal cord (PFU/ml)	Mortality (%)	Duration of virus recovery (days)	Time to death (days)
wt VSV		10 <sup>6</sup> , 10 <sup>7</sup>	100	2-3	2-3
<i>ts</i> G22	II	10 <sup>6</sup> , 10 <sup>5-6</sup>	100	7	5-10
<i>ts</i> G31	III	10 <sup>6</sup> , 10 <sup>5-6</sup>	100	7	5-10
<i>ts</i> G32	III	10 <sup>4</sup> , 10 <sup>3</sup>	40	5	7-19
<i>ts</i> G41	IV	10 <sup>4-5</sup> , 10 <sup>2</sup>	5-10	21	9-17, but most survived

oped hind-limb paralysis around 7 days postinfection which lasted for 21 to 28 days. Although most mice recovered, 5 to 10% remained paralyzed for the whole period of observation, i.e., 160 days. Pathologically, there occurred mononuclear inflammatory infiltrates in the gray and white matter of the spinal cord, leptomeningitis, neuronal dropout, reactive gliosis, and foci of primary demyelination (4). Viral titers in BALB/c mice infected with *ts* G41 were similar to those seen after VSV *ts* G32 infection and only 1% of those seen after *ts* G31 and *ts* G22 infection. Importantly, *ts* G41 could be recovered from both the brain and the spinal cord for up to 3 weeks after infection, and the isolated virus remained temperature sensitive (Table 3).

Several points emerge by contrasting different VSV *ts* infections. Although the clinical course of disease in *ts* G32 infected animals was similar to that of animals inoculated with *ts* G31 (also a mutant of complementation group III), the infections were pathologically different. Pathological alterations in mice infected with VSV *ts* G32 were similar to those observed in *ts* G41 infections. Furthermore, viral titers (Table 3) in mice infected with VSV *ts* G32 were also similar to those observed in *ts* G41 infection, a mutant of complementation group IV.

One may conclude, therefore, that both virological and neuropathological features produced by different *ts* mutants seem to be more dependent upon the characteristics of each particular mutant rather than upon the biochemical defects shared by all members of a complementation group. Our present study illustrates that within a given complementation group, various *ts* mutants may produce little, moderate, or extensive neuropathological disease.

Finally, we have begun to examine whether characteristics are shared by VSV *ts* mutants which possess neurovirulence. Clearly, the more neurovirulent *ts* mutants, such as G22 or G31, are capable of replicating to relatively high titer within the CNS, whereas less virulent *ts* mutants replicate to a more limited extent (Table 3). Nevertheless, even avirulent *ts* mutants, such as *ts* G21 and *ts* G23, appear to replicate as well as *ts* G32 or *ts* G41, yet produce almost no disease

(Table 3). Although the peak CNS viral titers achieved during infection with these mutants were similar to their more virulent *ts* counterparts, the duration of viral recovery was limited. Thus, the duration of viral persistence within the CNS, as well as the magnitude of viral replication, appears to be associated with the more neurovirulent VSV *ts* mutants.

Since our previous studies have shown a striking predilection of VSV for growth in neurons, we wondered whether neurovirulence was associated with different replicative patterns in neuronally-derived cells, as opposed to the standard cell systems used to assay the VSV viruses in vitro. It seemed reasonable to ask whether neurovirulent VSV *ts* mutants showed selective replicative advantage over their avirulent counterparts when infecting NB or glioma cells, particularly at semipermissive temperatures, a condition more nearly duplicating in vivo conditions. In an effort to examine this possibility, NB, and glioma cells, as well as BHK-21 cells, were infected with various group II and III VSV *ts* mutants. Although data for *ts* mutants G31, G22, and G41 are not shown, one could not distinguish by analyzing the growth curves which were the neurovirulent or avirulent mutants (data not shown; Figs. 1a through d). All *ts* mutants replicated about the same at permissive and semipermissive temperatures in NB, glioma, and BHK-21 cells. Clearly, these studies need to be repeated in organotypic cultures in which a better approximation of the in vivo situation exists than occurs with tumor-transformed cell lines. Nevertheless, it is clear from these studies that unique biological characteristics are associated with certain mutants within a complementation group and not shared with other *ts* mutants in the group. Thus, even though a common biochemical lesion is associated with all group III VSV *ts* mutants, neurovirulence is associated with yet another set of markers. The nature of these markers for neurovirulence is under active investigation in our laboratory.

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