The Uncoupled Relationship between the Temperature-sensitivity and Neurovirulence in Mice of Mutants of Vesicular Stomatitis Virus

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SUMMARY

Inoculation of wild-type (wt) VSV intracerebrally (i.c.) in Swiss weanling mice results in a rapidly fatal illness with death in two to three days. In contrast, i.c. inoculation of temperature-sensitive (ts) VSV mutants G31 and G22, but not ts G11 or ts G41, results in a more slowly progressive central nervous system (CNS) disease with distinct neurological signs. Studies undertaken to evaluate the neurovirulence of ts VSV mutants indicated that the ability of ts mutants to produce pathological changes in the CNS of mice appeared related to their ability to replicate to high titre in brain and spinal cord. However, replication of ts VSV mutants in brain alone was not sufficient to produce clinical illness. More importantly, the ability of ts VSV mutants to replicate at non-permissive temperatures in vitro did not appear to correlate with neurovirulence. VSV harvests from brains and spinal cords of mice infected with each of the ts mutants were temperature-insensitive. In spite of their temperature-insensitivity, the biological behaviour of viruses recovered from CNS tissues was, surprisingly, not that which was characteristic of revertant clones. Virus isolates recovered from infected CNS tissues, despite their temperature-insensitivity, behaved biologically like the original stocks of ts mutant virus. These data suggest that temperature-sensitivity is not directly correlated with the unique pathogenesis elicited by infection with ts VSV mutants.

INTRODUCTION

Temperature-sensitive (ts) mutants of many viruses have been described (Burge & Pfefferkorn, 1966; Simpson & Hirst, 1968; Mills & Chanock, 1971; Pringle & Duncan, 1971; Zygraich & Huygelen, 1973; Preble & Youngner, 1975). Study of these mutants has proved useful in delineating the genetics and biochemistry of virus replication. In addition to extensive in vitro studies directed at studying the effects of infection of cell culture systems with various ts mutants, considerable interest has been generated in assessing the capacity of ts mutants to either establish or maintain persistent virus infection in vivo or to alter the pathogenesis of disease associated with parental, wild-type (wt) virus infection (Clark & Koprowski, 1971; Simizu & Takayama, 1971; Clark & Wiktor, 1972; Haspel, Duff & Rapp, 1975; Rabinowitz, Dal Canto & Johnson, 1976; Tarr & Lubiniecki, 1796).

In earlier studies we focused our attention on the capacity of four ts mutants of vesicular stomatitis virus (VSV) to elicit disease in the central nervous system (CNS) of mice. In
general, the ts mutants showed clinical and histopathological features different from those produced by wt VSV (Rabinowitz et al. 1976). For example, after intracerebral (i.c.) inoculation of weanling outbred mice, wt VSV produces a fulminating illness leading to death within two to three days. Minimal clinical and histopathological changes in the CNS of mice accompanied CNS infection with wt VSV. In contrast to wt VSV, i.c. inoculation of ts G22 or ts G31 VSV produced a disease process which had an extended clinical course, distinct neurological signs and striking spongiform changes in the grey matter of the spinal cord (Dal Canto, Rabinowitz & Johnson, 1976a, b).

Although ts mutants of VSV have been shown to produce an infectious process that is quite distinct from the wt VSV, it is not known whether or not their altered growth at elevated temperatures is directly responsible for the clinical course of infection in the CNS. This study was primarily carried out to examine the relationship between temperature sensitivity and the biological activity of several strains of VSV ts mutants. To this end we have examined the ability of these viruses to replicate in the brain and spinal cord of mice, the nature of the viruses that are recovered from the infected tissues, as well as the ability of ts mutants propagated at 37 °C to cause CNS infection. In each case the growth kinetics and neurovirulence was compared to that previously observed with wt VSV.

METHODS

**Animals.** Outbred Swiss mice of both sexes, three to four weeks of age, were purchased from Scientific Products (Arlington Heights, Illinois). All mice were provided with food and water ad libitum.

**Virus infection.** Mice were injected with wt VSV or one of the following ts mutants: ts G11, ts G22, ts G31 and ts G41. Intracerebral inoculation was performed by injecting 0.03 ml through a 25-gauge needle inserted while animals were maintained under light anaesthesia. All virus suspensions were diluted in Hanks' balanced salt solution (HBSS).

**Cell culture lines.** BHK-21 cells were originally obtained from International Scientific Industries (Cary, Illinois) and grown to confluence in minimal essential medium with Earle's salts supplemented with 7 % foetal calf serum (FCS, virus-screened, Gibco, New York), 10 % (w/v) tryptose phosphate, 2 mm-L-glutamine, 0.1 % non-essential amino acids, 0.1 % minimal essential medium vitamins, and 100 units penicillin, 100 μg streptomycin, 10 μg gentamicin and 2.5 μg amphotericin B per ml. The cells were then maintained in the same medium except for the fact that 4 % FCS was used. These media will be referred to as either BHK-21 growth or maintenance medium.

**Viruses**

*Wild-type VSV.* Indiana strain VSV was obtained originally from the American Type Culture Collection and prepared as described previously (Rabinowitz et al. 1976). The stock wt VSV titre was $2 \times 10^9$ p.f.u./ml in BHK-21 cell monolayers at 37 °C.

*Ts mutants.* Ts G11, ts G22, ts G31 and ts G41 were generously provided by M. E. Reichmann (University of Illinois, Urbana, IL.). Each of these mutants was plaque-allowed by adding 0.1 ml of 10-fold dilutions of freshly thawed virus stock or tissue homogenate to each series of wells. Virus was allowed to adsorb for 60 min at 31, 37, or 39 °C in a 5 % CO₂ atmosphere, and wells were overlayed with 2 ml of
Eagle's basal medium supplemented with 5% FCS, 5% (w/v) tryptose phosphate, 2 mM-
L-glutamine, 100 units penicillin, 100 μg streptomycin, 2.5 μg amphotericin B and 0.7% 
Bacto-Agar (Difco Labs, Detroit, Mich.). Virus samples were then grown at both 31, 37 or 
39 °C for fluids containing ts mutants and wt VSV. After incubation for 24 h at the appro-
priate temperature, cultures were counterstained with a second overlay containing neutral red 
(1:9000). All samples were titrated in duplicate and plaques were counted 18 to 24 h after 
the second overlay by inverting the plates over an X-ray viewbox.

Organ preparation. Mice were anaesthetized with ether and exsanguinated by cardiac 
puncture. Brains were removed after sterile dissection through the scalp and calvarium and 
were obtained intact. Spinal cords were then removed by irrigation of the spinal canal with 
HBSS. This procedure yielded spinal cords quickly which were sterile and intact. Brains and 
spinal cords were then prepared as 10% (w/v) suspensions in HBSS and were stored frozen 
at -70 °C for subsequent virus titration.

Production and purification of defective-interfering (DI) particles of ts G31 VSV. Stock 
ts G31 VSV was passaged in 75 cm² flasks at a high multiplicity of infection (~ 100 p.f.u./ 
cell). After 48 h growth at 31 °C, supernatant fluids were harvested and clarified by low 
speed centrifugation to remove cell debris. Undiluted virus was then passaged a second time 
in 75 cm² flasks containing BHK-21 cells, harvested after 48 h, and clarified.

In order to prepare large volumes of third undilute passage ts G31 VSV for DI particle 
purification, second undilute passage ts G31 was used to infect twelve 32 oz prescription 
bottles containing confluent monolayers of BHK-21 cells. After infection for 48 h at 31 °C, 
the culture media was removed, pooled and clarified by centrifugation at 9000 g in a Sorvall 
GSA rotor for 10 min. The supernatant fluid was then centrifuged at 7000 g (av) in an IEC 
model 147 angle rotor for 2.5 h. The resulting virus pellets were pooled by resuspending 
them in 2 ml of TEN buffer (50 mM-tris-HCl, pH 7.4; 100 mM-NaCl; 5 mM-EDTA), sonicated 
for 20 s (Branson, Model W 140 D, at a power setting of 2), and the resulting opaque virus 
suspension carefully layered on 5 to 40% sucrose in TEN buffer gradients. The gradients 
were centrifuged at 150 000 g in an IEC SB 283 rotor for 40 min and the separated and clearly 
visible DI and standard (B) ts 31 virus bands were carefully removed by aspiration with 
Pasteur pipettes. Plaque assays in BHK-21 cells at 31 °C indicated that B-band ts G31 titre 
was 1 x 10⁸ p.f.u./ml, while DI band ts G31 contained 2.5 x 10⁵ p.f.u./ml. The DI particle 
concentration in the DI band was estimated to be approx. 2 x 10³ particles/ml using the 
spectrophotometric method of Huang & Wagner (1966) and the mol. wt. of ts G31 (DI) RNA 
as determined by Leamnson & Reichmann (1974).

RESULTS

Effect of ts mutants on survival

The initial experiments compared the susceptibility of weanling Swiss mice to infection 
with wt and ts mutants of VSV. Groups of six to ten mice were inoculated i.c. with log10 
dilutions of each virus preparation, and the LD₅₀ was determined by the method of Reed & 
Muench (1938). Table 1 compares the amounts of virus required to cause death from 
infection when the five virus preparations were administered by the i.c. route. The LD₅₀ for 
wt VSV was 7.5 p.f.u., while ts G11 and ts G41 were avirulent. The LD₅₀ for ts G22 was 
1 x 10⁴ p.f.u. and 5 x 10² p.f.u. for ts G31. Although not shown, all ts mutants and wt VSV 
failed to produce illness when administered intraperitoneally even in doses as large as 10⁷ 
p.f.u.

The effect of each virus preparation on time of death is shown in Fig. 1. Mice inoculated
Table 1. Lethality of wild-type VSV and ts mutants for three- to four-week-old Swiss mice after intracerebral inoculation

<table>
<thead>
<tr>
<th>VSV</th>
<th>Complementation group</th>
<th>LD$_{50}$ (p.f.u./mouse)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>ts G11</td>
<td>I</td>
<td>$&gt;10^7$</td>
</tr>
<tr>
<td>ts G22</td>
<td>II</td>
<td>$1\times10^4$</td>
</tr>
<tr>
<td>ts G31</td>
<td>III</td>
<td>$5\times10^2$</td>
</tr>
<tr>
<td>ts G41</td>
<td>IV</td>
<td>$&gt;10^7$</td>
</tr>
<tr>
<td>wt</td>
<td>—</td>
<td>$0.75\times10^1$</td>
</tr>
</tbody>
</table>

*Ts mutants were quantified by plaque assays in BHK-21 cells at 31 °C. wt VSV at 37 °C. Mice were observed daily for 21 days for signs of illness and death.

Fig. 1. Survival of three- to four-week-old Swiss mice after i.c. inoculation of wt VSV or ts VSV mutants. For wt VSV 24 mice were injected with $2.5\times10^2$ p.f.u./animal; for ts G31, 33 mice with $1.0\times10^4$ p.f.u./animal; for ts G22, 20 mice with $1.0\times10^4$ p.f.u./animal; for G11, 16 mice with $2.5\times10^6$ p.f.u./animal; for ts G41, 30 mice with $2.5\times10^6$ p.f.u./animal. - - -, wt VSV; — — —, ts 31; — — —, ts 22; — — —, ts 41; — — —, ts 11.

with wt VSV in doses ranging from $2.5\times10^2$ p.f.u. to $10^6$ p.f.u. died within four days of i.c. infection and 50% of the mice died within 1-4 days of inoculation. Ts G22 killed 50% of the mice within 6-2 days, while ts G31 killed 50% of the mice by 5-5 days after infection. In addition, following i.c. administration of ts G22, approx. 5% of the mice survived.

In contrast to the neurovirulent ts mutants described above, i.c. infection with ts G11 and ts G41 was invariably benign even when doses as large as $10^7$ p.f.u. were used.

Relationship between plaquing efficiency of ts mutants at various temperatures and neurovirulence

Although i.c. infection of mice with certain ts VSV mutants has been shown to elicit a unique spongiform myelopathy (Rabinowitz et al. 1976), the relationship between virus growth and the ability of these viruses to elicit this neuropathology has not been defined. In an effort to determine if virulence for mice correlated with the ability of the ts mutants to
grow at non-permissive temperatures, ts mutants were plaqued in BHK-21 cells at 31, 37, and 39 °C. Table 2 depicts the plaquing of ts mutants in BHK-21 cells at both permissive (31 °C), semi-permissive (37 °C) and non-permissive temperature (39 °C). It is notable that ts G41, a relatively avirulent mutant, plaques only 20-fold less at 37 and 39 °C than at 31 °C (Table 2). In contrast, both neurovirulent ts mutants, ts G22 and ts G31, plaque over three logs less at 37 and 39 °C than at 31 °C. Ts G11, another avirulent mutant, plaques over five logs less at 37 and 39 °C than at 31 °C (Table 2). Thus no direct correlation existed between the ability of ts mutants to plaque in vitro at 37 or 39 °C and their virulence for mice.

**Growth of wt and ts VSV in vivo**

Since it did not appear that virulence for mice correlated with ability of the ts mutants to replicate at non-permissive temperatures in vitro, it was next considered useful to compare the growth of wt VSV and ts mutants in brains and spinal cords as a function of time after infection. It seemed important to determine whether all of the ts mutants replicated in vivo in the CNS and, if so, for how long. All CNS virus isolates were then plaqued in BHK-21 cells at both permissive and semi-restrictive temperatures. Plaquing of in vivo virus isolates was done routinely at 37 °C because the rectal temperature of the mouse was determined to be 37.04 ± 0.5 °C. In several experiments in vivo isolates were plaqued at 31, 37 and 39 °C.
Growth of wt VSV in brain and spinal cords of infected mice was very rapid. By 24 h after i.c. inoculation of $1 \times 10^5$ p.f.u. wt VSV, titres of VSV in brain were between $10^6$ and $10^7$ p.f.u./ml. At 48 h after infection, brain titres were approx. $10^7$ to $10^8$ p.f.u./ml. Spinal cord titres of wt VSV followed those of VSV isolates observed in brains. As anticipated, wt VSV isolates plaqued equally well at 31, 37 and 39 °C.

After inoculation of $1 \times 10^4$ p.f.u. ts G31 i.c., brain titres initially declined to low levels 6 h post infection, then rose so that by one day after infection approx. $1 \times 10^5$ p.f.u. ts G31/ml brain were recovered (Fig. 2). Two days after infection, ts G31 virus titres in brain were about $10^6$ p.f.u./ml and remained at that level for several days after which they declined slightly. Although not shown, ts G31 virus was usually not detected by eight days after infection. Spinal cord titres of ts G31 closely followed brain titres with the following exceptions: (1) ts G31 virus was not recovered from spinal cords until three days after infection, and (2) ts G31 virus titres in spinal cords reached a peak of $1 \times 10^5$ p.f.u. four days after infection and declined significantly thereafter (Fig. 2). Again, although not shown, no ts G31 was recovered from spinal cords eight days after i.c. inoculation. Finally, it is important to recognize that both ts G31 brain and spinal cord isolates plaqued equally well at either 31 or 37 °C (Fig. 2).

Growth of ts G22 was next assayed in CNS tissues of infected mice (Fig. 3). In these experiments, $1 \times 10^4$ p.f.u. ts G22 was injected i.c. and the growth of ts G22 virus in CNS tissues resembled the kinetics seen after infection with ts G31. Thus, after declining to low levels 6 h after i.c. inoculation, ts G22 virus titres rose to a plateau 24 to 48 h after infection. Titres ranged between $10^5$ and $10^6$ p.f.u./ml brain for the first five days of infection and then declined so that by six days, brain titres were about $1 \times 10^4$ p.f.u./ml (Fig. 3). As in the case of ts G31, spinal cord titres of ts G22 closely followed the development and titres of ts G22 recovered from infected brain (Fig. 3). Ts G22 was first detected in spinal cords two days after infection, rose to peak values of $1 \times 10^6$ p.f.u./ml three to four days after infection, and declined significantly after six days. No virus was recovered from either brain or spinal cord by eight days after infection. Ts G22 CNS virus isolates were also found to be temperature insensitive (Fig. 3).
In contrast to the growth of ts G31 and ts G22, the replication of ts G11 (Fig. 4) and ts G41 (Fig. 5) was considerably less in both the brain and cord. Of note also is the fact that ts G11 brain and spinal cord isolates were temperature-insensitive. Even avirulent mutants such as ts 11, therefore, are both capable of replicating in vivo, and apparently losing the temperature-sensitivity originally expressed in vitro. In contrast to the other mutants, however, brain isolates of ts G41 plaqued 1 to 2 logs lower when assayed at 37 °C, suggesting some preservation of temperature-sensitivity of the brain isolates. Although difficult to explain, it was interesting to note that spinal cord isolates of ts G41 appeared temperature-insensitive (Fig. 5). Additional experiments were performed titrating brain isolates of all ts VSV infected mice at 39 °C. Results of these experiments, although not depicted, indicated that ts G22 and ts G31 brain and spinal cord isolates plaqued equally well at 31, 37 and 39 °C.
Although ts G11 and ts G41 plaqued equally at 37 and 39 °C, titres were approx. 1 to 2 logs higher at 31 °C. Thus, it does not appear as if the temperature-insensitivity of the brain and spinal cord isolates are the result of utilizing the semi-restrictive temperature (37 °C) rather than the restrictive (39 °C) temperature for plaquing.

**Biological behaviour of CNS isolates of ts G22 and ts G31**

Since brain and spinal cord isolates of ts G22 and ts G31 infected mice were temperature-insensitive, we reasoned that the neurovirulence of these mutants might be readily explained by either the generation of revertant clones under *in vivo* conditions, or the presence of revertants in the original ts population. If temperature-insensitivity indicated that neurovirulence resulted from selection of revertants, then brain isolates of ts G22 and ts G31 infected mice should behave biologically as wt virus. To test this hypothesis, comparable amounts (10^4 p.f.u.) of wt VSV as well as brain pool-derived and original ts mutant grown at 31 °C in tissue culture were injected i.c. and survival recorded. Brain pools of ts G22 were prepared four days after infection when titres of ts G22 were at their highest levels (Fig. 3). When comparable amounts of all three virus preparations were injected i.c., the brain-derived isolate of ts G22 behaved biologically more like the ts G22 prepared in tissue culture, and not like wt VSV (Fig. 6).

The ts G31 recovered from the brains of infected mice also behaved biologically much more like the original ts mutant than wt VSV (Fig. 7). We found that 10^4 p.f.u. wt VSV killed 50 % of the mice in 1·2 days, whereas 10^4 p.f.u. brain-derived ts G31, obtained four days after infection, killed 50 % of the mice in 4·3 days, and original tissue culture prepared ts G31 killed 50 % of the mice in 5·5 days (Fig. 7).

This phenomenon was not restricted to ts mutants that were recovered from the CNS of mice. When each ts mutant was grown at 37 °C *in vitro*, and then inoculated i.c. into mice,
Fig. 7. Survival of mice after i.c. inoculation of wt VSV, ts G31 isolated from brain, or original ts 31 prepared in BHK-21 cells at 31 °C. Groups of seven to ten mice were inoculated i.c. with 1·0 x 10^4 p.f.u. of each virus preparation and survival recorded. Ts G31 (TC) represents original tissue culture grown ts G31 VSV, while ts G31 (BP) represents ts G31 obtained from three brains four days after i.c. inoculation of ts G31. ——, ts 31 (BP); ———, ts 31 (TC); ———, wt VSV.

Table 3. Lethality for three- to four-week-old Swiss mice after intracerebral inoculation of various ts VSV mutants passaged in BHK-21 cells at 37 °C

<table>
<thead>
<tr>
<th>Ts mutant</th>
<th>Virus inoculum (p.f.u.)</th>
<th>Days at which individual mice died</th>
</tr>
</thead>
<tbody>
<tr>
<td>I G11</td>
<td>1·5 x 10^6</td>
<td>7, 8; 5 survived*</td>
</tr>
<tr>
<td>II G22</td>
<td>6 x 10^3</td>
<td>5, 6, 6, 6, 6, 7, 8</td>
</tr>
<tr>
<td>III G31</td>
<td>8·25 x 10^3</td>
<td>5, 5, 5, 5, 6, 7</td>
</tr>
<tr>
<td>IV G41</td>
<td>1·5 x 10^6</td>
<td>6, 7, 7, 7, 11; 2 survived*</td>
</tr>
</tbody>
</table>

* All mice remained clinically well throughout 21 days of observation. Titres of ts mutants were determined in BHK-21 cells at 37 °C.

Table 4. Lethality of various doses of wt VSV for three- to four-week-old Swiss mice after intracerebral inoculation

<table>
<thead>
<tr>
<th>Number of mice</th>
<th>Virus dose (p.f.u.)</th>
<th>Days after infection at which individual mice died</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>10^6</td>
<td>2, 2, 2, 2, 2, 2, 3, 3</td>
</tr>
<tr>
<td>6</td>
<td>10^4</td>
<td>2, 2, 2, 2, 2, 3</td>
</tr>
<tr>
<td>6</td>
<td>10^3</td>
<td>2, 2, 2, 2, 2, 3</td>
</tr>
<tr>
<td>5</td>
<td>1·5 x 10^4</td>
<td>3, 3, 3, 3, 4</td>
</tr>
<tr>
<td>6</td>
<td>0·75 x 10^3</td>
<td>3, 3; 4 survived*</td>
</tr>
</tbody>
</table>

* Mice remained clinically well throughout 21 days of observation.
the survival rates and the length of time to death were comparable to that observed after inoculation of the original ts mutants grown at 31 °C (Table 3). The only exception to this was that an apparent increase in lethality was observed when avirulent ts mutants G11 and G41 were used. When ts G11, grown at 37 °C was inoculated i.c. two out of seven mice died, whereas ts G11 grown at 31 °C never caused lethal infection (Fig. 1 and Table 3). Similarly, ts G41 passaged at 37 °C appeared to be more lethal for mice than ts G41 grown in vitro at 31 °C. It is interesting that although virulence appeared somewhat enhanced, time until death remained unaffected (Table 3). Moreover, each ts mutant passaged at 37 °C in vitro was found to be temperature-insensitive. Thus, in these experiments as in earlier studies using brain-derived isolates of ts mutants, an apparent dissociation existed between the presence of the phenotypic markers for temperature-sensitivity and biological neurovirulence.

Finally, if wt VSV was present in low titre in the original ts inoculum, and resulted in delayed death because wt VSV required a longer time to reach titres sufficient in vivo to kill, then i.c. inoculation of very low doses of wt VSV should result in survival curves comparable to those seen after inoculation of ts G22 or ts G31 brain isolates. However, low doses of wt VSV, 15 p.f.u. or 7.5 p.f.u., lengthened time to death only by one or two days (Table 4). Furthermore, none of the mice surviving for three to four days ever developed neurological signs comparable to those observed after inoculation of ts mutants (Rabinowitz et al. 1976).

**Relationship of CNS infection produced by ts G31 to presence of DI particles**

Doyle & Holland (1973) have reported that i.c. inoculation of massive doses of DI particles with wt VSV produced a slowly progressive neurological illness similar to that observed with ts VSV mutants. It was, therefore, important to determine if the clinical progress of the CNS disease, produced by ts mutants, resulted from the presence of DI particles in the original ts inoculum. To explore this possibility, undiluted third passage ts G31 VSV prepared in BHK-21 cells was subjected to sucrose density gradient purification as described in Methods. Table 5 depicts the results when mice were injected i.c. with either gradient purified ts G31 or similar amounts of ts G31 mixed with homologous DI particles. It can be seen that ts G31 VSV, substantially free of homologous DI particles, produces CNS infection culminating in death in a manner comparable to that previously described for ts G31 (Fig. 1). In contrast, when large numbers of DI particles are present along with standard ts G31 VSV, the majority of mice develop no illness. Thus, it does not appear that the slowly progressive CNS infection seen with ts G31 VSV is the result of the presence of homologous DI particles in the original inoculum.

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**Table 5. Mortality for three- to four-week-old Swiss mice after intracerebral inoculation of gradient purified ts G31 DI particles and standard ts G31 VSV**

<table>
<thead>
<tr>
<th>Number of mice</th>
<th>ts G31 virus inoculum (p.f.u.)</th>
<th>Days after infection at which individual mice died</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>$3 \times 10^4$</td>
<td>5, 5, 5, 6, 7, 7, 7, 8</td>
</tr>
<tr>
<td>20</td>
<td>$7.5 \times 10^4$ plus $6 \times 10^{11}$ DI-ts G31 particles</td>
<td>8, 10; 18 survived*</td>
</tr>
</tbody>
</table>

*Mice remained clinically well throughout 21 days of observation.*
\textbf{DISCUSSION}

Inoculation of wt VSV in three- to four-week-old Swiss mice produces a fulminating disease with death in two days unaccompanied by any specific neurological signs. Inoculation of \textit{ts} G22 or \textit{ts} G31, but not \textit{ts} G11 or \textit{ts} G41 results in a disease characterized by a slower course, appearance of severe paralysis of the hindlimbs beginning at four days post-infection and death generally around seven to eight days (Rabinowitz \textit{et al.} 1976). Equally important are the differences in pathological changes produced by wt VSV and \textit{ts} mutants of G31 and G22. While wt VSV produces minimal histopathological changes in brain and spinal cord consistent with mild encephalitis, \textit{ts} G31 and \textit{ts} G22 induce extensive spongiform changes confined to the grey matter of the spinal cord (Dal Canto \textit{et al.} 1976a, b).

In our studies, two VSV \textit{ts} mutants were neurovirulent: \textit{ts} G22 and \textit{ts} G31 (Fig. 1). These \textit{ts} mutants were members of complementation group II and III respectively. Only \textit{ts} G11, a member of complementation group I, appeared completely avirulent (Fig. 1). Nevertheless, previous reports (Stanners & Goldberg, 1975), have suggested that \textit{ts} 1026, a member of complementation group I, is capable of being neurovirulent. Thus, it does not appear that neurovirulence is the property of any single complementation group or that all \textit{ts} mutants within a single genetic complementation group exhibit the same degree of virulence. The implications of this are that no single biochemical defect, common to any given complementation group, appears to be a prerequisite for neurovirulence.

Of considerably more importance in an understanding of neurovirulence are the kinetics of \textit{ts} mutant replication \textit{in vivo}. Our studies clearly show that both \textit{ts} G22 and \textit{ts} G31 are capable of replicating \textit{in vivo} to high titres in both brain and spinal cord (Fig. 2 and 3). In contrast, avirulent mutants \textit{ts} G11 and \textit{ts} G41 replicate to only a limited extent in brain and to low titre for a short period in the spinal cord (Fig. 4 and 5). It appears that \textit{ts} mutants capable of replicating to high titres and for a prolonged period in spinal cord, result in CNS disease and death. The \textit{ts} mutants, such as \textit{ts} G11 and \textit{ts} G41 which are only capable of replication in brain, are avirulent. Interestingly, even neurovirulent \textit{ts} G22 or \textit{ts} G31 never achieve titres in CNS tissues comparable to those found with wt VSV infection.

It is of interest to compare the growth characteristics of the \textit{ts} mutants \textit{in vivo} and \textit{in vitro}. It is apparent that the ability of the \textit{ts} mutants to replicate \textit{in vitro} in BHK-21 cells at 37 or 39 °C bears little relationship to their capacity to replicate \textit{in vivo} (Table 2 and Fig. 2 and 5). \textit{ts} G41, a relatively avirulent \textit{ts} mutant, plaques only 20-fold less at 37 and 39 °C than it does at 31 °C, but grows to only a limited extent in brain and spinal cords of infected mice (Table 2 and Fig. 5). \textit{ts} G22 or \textit{ts} G31, on the other hand, plaque at least three logs less at 37 and 39 °C than at 31 °C, but grow to high titre \textit{in vivo} in both brain and spinal cord (Table 2 and Fig. 2 and 3). Thus, the ability of these viruses to grow \textit{in vitro} at 37 and 39 °C is not directly related to the capacity of a given \textit{ts} mutant to be neurovirulent.

All neurovirulent \textit{ts} mutant isolates derived from brain and spinal cord of infected mice were temperature-insensitive. Because of the temperature-insensitivity of the isolates, one of the first explanations offered for virulence related to the generation of revertant clones of wt VSV. There are at least three reasons why this does not seem an adequate explanation in our model system: (1) brain and spinal cord isolates of \textit{ts} G11 (Fig. 4) were temperature-insensitive and yet \textit{ts} G11 was avirulent; (2) inoculation of temperature-insensitive brain pool-derived \textit{ts} G22 and \textit{ts} G31 resulted in clinical disease and mortality quite comparable to that observed after inoculation of the original \textit{ts} mutant (Fig. 6 and 7) rather than with wt VSV; (3) inoculation of \textit{ts} VSV mutant passaged \textit{in vitro} at 37 °C, a procedure resulting in production of temperature-insensitive mutants, does not lead to infection comparable...
to that seen after inoculation of the original ts mutants propagated at 31 °C. However, the outcome of an infection at any particular temperature, in vivo or in vitro, can be influenced by the complex interaction of several factors. Such factors include the leakiness of the mutant (Pringle, 1970), generation of partial revertants and the subsequent interaction of ts virus and revertants with specific regard to selective advantages to either virus at non-permissive temperatures. In this regard, Youngner & Quagliana (1976) have recently reported that under in vitro conditions when mixtures of ts VSV and wt VSV are added to L-cell cultures, replication of ts virus predominates. Thus, it appears that, under conditions of mixed infection, ts VSV replicates at the advantage of wt VSV in a manner analogous to mixed DI-wt VSV infection. Finally, it is important to emphasize that the finding of temperature-insensitivity in isolates obtained in vivo from brain and spinal cord, does not, under the limitations of the assay system employed in these studies, rule out the presence of some ts mutant virus. It should be stressed, however, that the brain and spinal cord isolates behave phenotypically as if they were temperature-insensitive and yet act biologically as if they were temperature-sensitive.

While our data do not support the idea of a major role for revertants in the neurovirulence observed with certain ts VSV mutant in vivo, they do not exclude the possibility that partial reversion occurred during the pathogenesis of CNS infection. Interestingly, Stanners & Goldberg (1975) found that in newborn hamsters, ts G31, ts G22, ts G11 and ts G41 behaved biologically much like wt VSV, resulting in rapid death. In their experiments, the virulence of ts mutants of VSV in newborn hamsters was positively correlated with their tendency to generate revertants and with their leakiness in cultured hamster embryo fibroblasts at 37 °C. These observations serve to emphasize the contribution of the host to the pathogenesis of CNS infection.

It does not appear that interference, mediated by DI particles, is responsible for the alteration seen in CNS disease induced by ts VSV mutants. Although DI particle interference with virus-induced pathogenicity has been reported (Doyle & Holland, 1973; Holland & Villarreal, 1974), and the presence of DI particles can modify the clinical course of infection with ts VSV mutants (Table 5), our results indicate that the standard ts G31, free from DI particles, possesses the capacity to induce a typical ts mutant CNS infection. The possible role of DI particles generated during infection, as well as possible alternative forms of virus interference are being investigated in an attempt to explain the pathogenic potential of certain ts VSV mutants following CNS inoculation.

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ts-VSV CNS infection


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