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Neurovirulence Mutant of Vesicular Stomatitis Virus with an Altered Target Cell Tropism In Vivo

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Intracerebral infection of weanling Swiss mice with a temperature-sensitive (*ts*) mutant of vesicular stomatitis virus (VSV), *ts* pi364, resulted in a unique neuropathological syndrome not previously described with other VSV mutants. Mice infected with wild-type VSV died from an acute encephalitis characterized by neuronal necrosis and efficient virus replication in both brain and spinal cord. In contrast, with VSV *ts* pi364, the most prominent histopathological feature was destruction of the ependyma of the lateral ventricles. Virus antigen was also limited to the leptomeninges and the lateral ventricles. Infected mice survived and developed hydrocephalus. Replication of *ts* pi364 in the brain was 10- to 100-fold less than that of wild-type VSV, and appearance of virus in the spinal cord was delayed. VSV *ts* pi364 was isolated from mouse cells persistently infected with VSV. Another VSV *ts* pi mutant, isolated from the same persistent infection, behaved in vivo like wild-type VSV, even though both mutants were very similar in plaque size, reversion frequency, cut-off temperature, and synthesis of virus-specific proteins at semipermissive temperature. These results strongly suggest that VSV *ts* pi364 has a second, non-*ts* mutation which results in a restricted target cell range in vivo; wild-type VSV can infect both neurons and ependymal cells, whereas *ts* pi364 does not replicate in neurons.

Early studies with reovirus (8, 9) and measles virus (1, 10) showed that virus mutants could be used to investigate the virus- and host-specific factors involved in neurovirulence. Certain classes of temperature-sensitive (*ts*) virus mutants produced delayed or unusual neurological syndromes in infected animals. More recently, this experimental approach has also been used to study the biochemical basis for neurovirulence of other viruses.

Certain *ts* mutants of mouse hepatitis virus (11, 22) and vesicular stomatitis virus (VSV) (4, 5, 19, 30, 34) differ in neuropathogenicity in vivo compared with wild-type (WT) virus. Intracerebral infection of mice with either of two *ts* mutants of VSV, *ts* G22 (II) or *ts* G31 (III), resulted in extensive spongiform changes in the gray matter of the spinal cord which were not observed in mice infected with WT virus (19). Further investigation into the mechanism(s) involved in this altered neuropathogenesis showed that maturation of *ts* G31, a late mutant with a *ts* lesion in the M protein, was defective both in neurons in vivo and in infected neuroblastoma cell cultures incubated at 39°C, the nonpermissive temperature for the *ts* mutant (6, 12, 13). Whether a similar situation was also obtained with *ts* G22, an early mutant with a *ts* defect in one of the viral nucleocapsid proteins, was not reported. Virus isolated from mice infected with VSV *ts* G31 or *ts* G22 was no longer temperature sensitive but still behaved in vivo like the original *ts* mutants after injection of mice (20), suggesting that the *ts* phenotypic marker and the neurovirulence locus were independent in these mutants. This explained previous work which showed that various VSV *ts* mutants, all of which mapped in complementation group I, could behave very differently in infected animals (30).

Further investigation of the virus-cell interactions involved in VSV neuropathogenicity might be expedited if specific neurovirulence mutants were available. We present data in this report which indicate that a VSV mutant (*ts* pi364) which was isolated from mouse L cells persistently infected with VSV (36) is, in fact, such a mutant. VSV *ts* pi364 has a restricted target cell tropism in vivo which results in a unique neuropathological syndrome not previously described with other VSV mutants.

MATERIALS AND METHODS

Cells. Primary chicken embryo (CE) cells, mouse L cells, and the Vero line of monkey kidney cells were

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propagated in Eagle minimal essential medium (MEM) plus 4% calf serum. BHK-21 cells were grown in MEM containing 10% tryptose phosphate broth and 5% fetal calf serum.

Mice. Weanling (3 to 4 weeks old) female outbred Swiss Webster mice (Taconic Farms, Germantown, N.Y.) were used in all experiments.

Viruses. The virus used in these experiments was VSV Indiana strain. Virus titers, expressed as plaque-forming units (PFU) per milliliter, were determined on monolayers of CE fibroblast cells incubated at 34°C, the permissive temperature for VSV *ts* mutants. The nomenclature for virus is as follows: (i) WT VSV, VSV Indiana strain; (ii) VSV *ts* G11, isolated after chemical mutagenesis of stocks of WT VSV (18) and obtained from R. Wagner, Charlottesville, Va. (Plaque-purified stocks of VSV *ts* G11 were used.); (iii) VSV *ts* pi mutants 364 and 421, recovered from mouse L cells persistently infected with WT VSV (L_{VSV} cells) (36). The persistently infected cultures were maintained at 37°C. *ts* pi364 was isolated 7 days after initiation of persistence, whereas *ts* pi421 was isolated 10 days after infection. Both *ts* pi clones produce small plaques on CE cells at 34°C and are temperature sensitive at 39.5°C.

All virus stocks were grown at 34°C in BHK-21 cells by using a low multiplicity of infection (<.01 PFU per cell) of plaque-purified virus which had been amplified once at 34°C in CE cells. This procedure was used to minimize the level of defective interfering particles in virus preparations injected into mice.

Infection of mice. Intracerebral inoculation of mice was done by injecting 0.03 ml of virus in MEM into the right side of the brain of lightly anesthetized mice with a 26-gauge, 3/8-inch (.9525-cm) needle. Control animals received MEM alone by the same route. Mice were sacrificed by cardiac puncture under light chloroform anesthesia, followed by immediate removal of the brain and spinal cord for viral and pathological studies. Spinal cords were removed by irrigation of the spinal canal with MEM.

To prepare tissue homogenates for virus assay, brains or spinal cords from at least two mice were pooled. Suspensions (10% [wt/vol]) of the tissues in MEM were made by three cycles of freezing and thawing followed by disruption of tissue fragments by sonic oscillation for 2.5 to 3 min. After samples were removed for infectivity determinations, the homogenates were stored at −70°C.

Localization of virus antigens by immunofluorescence. Mice were sacrificed by cardiac puncture followed by immediate removal of the brain, including brainstem and a short section of cervical spinal cord. This tissue was then embedded in Tissue-Tek II O.C.T. compound (Miles Laboratories, Inc., Elkhart, Ind.) and quickly frozen. Sections (4 to 8 μm thick) were prepared, fixed in acetone at room temperature for 3 to 5 minutes, and air dried. Virus antigens were localized with an indirect immunofluorescence procedure utilizing rabbit hyperimmune anti-VSV serum and fluorescein-conjugated goat-anti-rabbit 7S globulin (Meloy, Springfield, Va.). Slides were examined by using an epifluorescence microscope (ultrastar; American Optical Corp., Buffalo, N.Y.). Tissue sections from mock-infected mice served as negative controls, whereas brain sections from moribund mice infected with WT VSV served as positive controls in all experiments.

Assay of interferon in brain homogenates and serum of infected mice. Samples of the 10% brain homogenates used for virus infectivity assay were clarified first at 3,000 rpm for 20 min and then at 10,000 rpm for 45 min. Each sample was adjusted to pH 2 by adding approximately 50 μl of 1 N HCl and incubated at 4°C for 48 h to inactivate infectious virus; samples were then returned to pH 7.2 by addition of an appropriate amount of 1 N NaOH. Any precipitate formed during this procedure was removed by centrifugation at 10,000 rpm for 45 min. This clarification procedure did not result in nonspecific loss of interferon from the brain homogenates. In a reconstitution experiment, a 10% homogenate of an uninfected brain was prepared in medium containing 10 U of interferon, and the homogenate was then clarified as described above. The processed sample and the original interferon-containing medium which was used to prepare the homogenate were then assayed. Both samples had the same titer.

Serum samples were clarified at 2,500 rpm for 30 min, and a sample was incubated on CE cell monolayers for 48 h at 34°C to screen for the presence of virus. A few serum samples contained virus as indicated by cytopathic effects in the CE cells. These samples, from mice infected with WT virus or *ts* pi421 and sacrificed 24 to 48 h after infection, were incubated at pH 2 as described above. Virus-free serum samples were assayed without treatment at pH 2.

Clarified brain homogenates and serum samples were assayed for the presence of interferon by using a semi-micro system which measures inhibition of VSV-induced cytopathogenicity in L cell cultures (28). The interferon titer is the reciprocal of the highest dilution of sample which protects at least 50% of the cells. A standard reference interferon sample was included in each assay; this standard varied within a twofold range. A 1-U amount of interferon in these assays was usually the equivalent of 2 U of a reference mouse interferon standard provided by the Antiviral Substances Program of the National Institute of Allergy and Infectious Diseases.

Screening of virus populations for temperature sensitivity. Tissue homogenates (10%) were assayed on CE cells. Plaques were then picked from terminal dilution plates into 1.5 ml of MEM, and the virus was allowed to elute from the agar overnight at 4°C (37). The virus clones were screened for temperature sensitivity by adding 0.1 ml of the virus-containing fluid to CE cell monolayers in wells of 24 welled trays (Linbro Chemical Co.); duplicate welled trays were then incubated at 34°C and 39.5°C. Virus-induced cytopathogenicity was scored after 48 h of incubation; lack of cytopathogenicity at 39.5°C correlated extremely well with restriction of virus replication (37). Supernatant fluids were also harvested from wells at both temperatures and plaque assayed at 34°C, the permissive temperature. Efficiency of yield values (39.5°C yield/34°C yield) were then calculated for each virus clone screened in the above manner. A value of 10^{-3} or less was indicative of temperature sensitivity. WT virus was included in every screening

test, and the efficiency of yield ranged from 0.5 to 1.0 in different experiments.

Polyacrylamide gel electrophoresis. Virus structural proteins were analyzed by using the Cleveland et al. (3) technique of limited proteolysis. [^{3}H]leucine-labeled virus grown in BHK-21 cells at 34°C was purified on rate zonal (10 to 40%) and isopycnic (10 to 50%) sucrose gradients and suspended in 0.01 M tris(hydroxymethyl)aminomethane(Tris)–0.1 M NaCl–0.001 M ethylenediaminetetraacetate (EDTA) (pH 7.4). Purified virus was mixed 1:1 with sample buffer described by Laemmli (15), heated in a boiling water bath for 3 min, and cooled in ice. Virus proteins were separated on 10% polyacrylamide slab gels prepared by the method of Laemmli (15) and visualized after staining with Coomassie brilliant blue followed by brief destaining by the method of Cleveland et al. (3). Some acid hydrolysis of the N protein apparently occurred during this destaining period; N protein run in the Cleveland et al. system without enzyme showed two fragments in addition to undigested N. The visualized protein bands were cut from the gel and stored at −20°C. The thawed gel bands were inserted into the sample wells of a polyacrylamide slab gel with a 4-cm long, 3% acrylamide stacking gel and a 15% acrylamide running gel. Each well was then overlaid with 10 μl of layering solution (0.125 M Tris [pH 7.0]–0.1% sodium dodecyl sulfate–0.001 M EDTA–10% glycerol–bromophenol blue marker dye) or layering solution containing various concentrations of *Staphylococcus aureus* V-8 protease (Miles Laboratories, Inc.) or chymotrypsin (Sigma Chemical Co., St. Louis, Mo.). Electrophoresis was carried out at 50 V until the bromophenol blue marker was approximately 0.5 cm from the bottom of the stacking gel, at which time electrophoresis was discontinued for 30 min to allow partial proteolytic digestion of the proteins (3). Electrophoresis was continued at 100 V until the marker dye was near the bottom of the running gel. The gel slabs were soaked for 1 to 2 h in En^{3}Hance solution (New England Nuclear Corp., Boston, Mass.), rinsed overnight in distilled water, dried onto filter paper, and exposed to Kodak X-omat X-ray film.

RESULTS

Origin and neurovirulence of VSV *ts* pi mutants. The VSV mutants used in this investigation are described in Table 1. Cloned WT VSV Indiana grew equally well at 34 and 39.5°C and was extremely lethal for weanling (3 to 4 weeks old) Swiss mice inoculated intracerebrally. All infected animals receiving greater than 2 PFU of WT VSV died by 72 h after infection. VSV *ts* pi364 and *ts* pi421 were both isolated from one line of L cells persistently infected with VSV (36). The L_{VSV} culture was initiated in 1975 by infecting cells with WT VSV which had been serially passed in BHK cells and contained approximately 1,000 defective interfering virus particles for each infectious particle. Under these conditions, there was a rapid spontaneous selection of *ts* virus mutants (36). The *ts* pi364 mutant was cloned from culture fluids harvested from the L_{VSV} cells 7 days after infection, whereas the *ts* pi421 mutant was isolated from fluids harvested from the same culture 3 days later. VSV *ts* pi364 and *ts* pi421 were therefore derived from the same parental WT virus. Both virus clones produce small plaques in CE cells at 34°C, and neither virus clone synthesizes a significant amount of virus-specific ribonucleic acid (RNA) in infected cells at the nonpermissive temperature (36; Preble, unpublished data).

However, as shown in Table 1, VSV *ts* pi421 was almost as virulent for mice as WT virus, except that infected mice died 24 to 48 h later than those given WT VSV. In contrast, *ts* pi364 produced very little clinical disease in infected mice even at extremely high doses of virus. In agreement with previous results (4, 5, 19, 30, 34), VSV *ts* G11, a chemically induced mutant, also was avirulent for mice. Several large plaque and non-*ts* multistep revertants of VSV *ts* pi364 were similar to WT VSV in neurovirulence; data for one representative revertant clone, *ts*$^+$ pi364 R1, is included in Table 1.

Examination of tissue sections stained with hematoxylin and eosin revealed that the histopathological lesions in the brains and spinal cords of mice infected with WT VSV and *ts* pi421 were very similar but that infection of mice with the *ts* pi364 mutant resulted in a unique neuropathological picture. WT VSV and *ts* pi421 produced acute encephalitis with perivascular infiltration of inflammatory cells; acute meningitis was also seen (Barmada et al., submitted for publication). Neuronal necrosis was advanced in both the brain and spinal cord (Fig. 1A) by 48 to 72 h after infection, when animals were moribund. Necrosis of ependymal cells in the brain and especially in the central canal of the spinal cord (Fig. 1A) was also apparent as compared with mock-infected control mice (Fig. 1B).

In contrast, no encephalitis was seen at any time after intracerebral infection of mice with VSV *ts* pi364. The most prominent histopathological finding was severe damage to ependymal cells in the brain, especially in the lateral ventricles. By 5 to 6 days after infection, dilation of the lateral ventricles was already apparent (Barmada et al., submitted for publication). By 3 to 4 weeks after infection with VSV *ts* pi364, striking hydrocephalus with massive distention of the lateral ventricles (Fig. 1C) was evident. At this time, the aqueduct was almost completely denuded of ependymal cells (Fig. 1D).

Table 2 summarizes the differences in neuropathology caused by WT VSV, *ts* pi421, and *ts* pi364. WT virus and the neurovirulent *ts* pi421 mutant produced a rapidly fatal acute encephalitis in which both neurons and ependymal cells

TABLE 1. *Neurovirulence of WT VSV and VSV ts pi mutants*

Virus clone	Origin of virus mutants	Intracerebral LD_{50}[a] (dose per mouse)	Disease syndrome
WT VSV Indiana		0.5 PFU	Preterminal lethargy, ruffled fur, convulsions, and paralysis; all infected animals dead by 3 days
VSV *ts* pi364	Isolated from L_{VSV} "I" line (36) 7 days after initiation with WT VSV Indiana	$>2 \times 10^7$ PFU	Humped posture and/or hind leg paralysis in 10–15% of mice receiving $>10^6$ PFU; otherwise, no clinical signs of disease
VSV *ts* pi421	Isolated from L_{VSV} "I" line (36) 10 days after initiation	20 PFU	Similar to that caused by WT VSV; infected animals died between 3 and 5 days postinfection
VSV ts^+ pi364 R1	ts^+ and large plaque multistep revertant of *ts* pi364	1 PFU	Similar to that caused by WT VSV
VSV *ts* G11	Chemically induced group I *ts* mutant (18)	$>10^6$ PFU	Hind leg paralysis in 5% of mice infected with $>10^6$ PFU; otherwise, no clinical signs of disease

[a] LD_{50}, The 50% lethal dose.

in the brain and the spinal cord were targets for virus infection (Barmada et al., submitted for publication). However, in mice infected with VSV *ts* pi364, the lack of encephalitis and nerve cell destruction allowed the animals to survive, and the extensive damage to ependymal cells resulted in hydrocephalus. Hydrocephalus was found in >90% of infected animals which received at least 100 PFU of VSV *ts* pi364 and was not observed in animals surviving infection with either WT VSV or *ts* G11, other prototype VSV *ts* mutants, other *ts* pi viruses, or very low doses of WT VSV (Preble and Barmada, unpublished data). All apparently healthy mice which survived infection with >100 PFU of VSV *ts* pi364 had high levels of neutralizing antiviral antibody (1:100 to >1:2,000) which remained stable for at least 3 months.

***ts* defects of *ts* pi364 and *ts* pi421.** We investigated other virus-specific properties that might correlate with the neuropathogenicity of VSV *ts* pi364 and *ts* pi421. Both VSV *ts* pi364 and *ts* pi421 are very early mutants. VSV *ts* pi364 is RNA^- at 39.5°C (36; Preble, unpublished data); the *ts* mutation of this *ts* pi mutant maps in complementation group I (36) and therefore presumably affects the L protein. VSV *ts* pi421 is also RNA^- at the nonpermissive temperature; *ts* pi421-infected BHK-21 cells incubated at 39.5°C produce less than 5% of the actinomycin D-resistant RNA synthesized in cells infected with WT VSV. However, the *ts* defect of *ts* pi421 maps in complementation group IV, suggesting that the *ts* defect may be in the N protein (14, 16). VSV *ts* pi421 complements well with prototype *ts* mutants from groups I, II, III, and V (complementation index, 9.6 to 343), but does not complement *ts* G44 (IV) (complementation index, 1.4). VSV *ts* pi364 and 421 also complement efficiently (complementation index, 655). Previous work (20, 30) has conclusively shown, however, that there is no apparent correlation between the complementation group of a given VSV *ts* mutant and the degree of neurovirulence of that mutant in mice. More attention is given to this matter in Discussion below.

The frequency of non-*ts* revertants in independently subcloned stocks of *ts* pi364 averaged $<10^{-6}$, and a similar value was also found with subclones of *ts* pi421. In addition, the yield of infectious virus per infected CE, L, or BHK-21 cell was similar for both *ts* pi mutants (data not shown). These parameters therefore do not appear to be involved in the difference in neuropathogenicity demonstrated by these mutants.

Replication of VSV *ts* pi mutants in the brain and spinal cord. Preliminary cut-off temperature experiments with *ts* pi364 and *ts* pi421 in mouse L cells showed that both mutants replicated to the same extent (10 to 20% of that at 34°C) at mouse body temperature, 37 to 38°C

(data not shown). However, replication of *ts* G11 was inhibited over 100-fold in this temperature range, suggesting that lack of virulence was due to lack of virus growth in vivo. To quantitate virus replication in vivo, mice were infected intracerebrally, and at various times, infectivity in 10% homogenates of brain or spinal cord was determined. Figure 2A (closed circles) shows that the concentration of WT virus rose rapidly until 64 h postinfection, when most of the infected animals had died or were moribund. The growth curve of *ts* pi421 in the brain was very similar to that of WT virus, except that viral replication began after a longer lag period (Fig. 2A, open triangles). These results, therefore, agreed with the delayed time of death seen after infection with *ts* pi421 (Table 1). The virus population present late in infection with *ts* pi421 was temperature sensitive; a more detailed examination of the characteristics of this virus

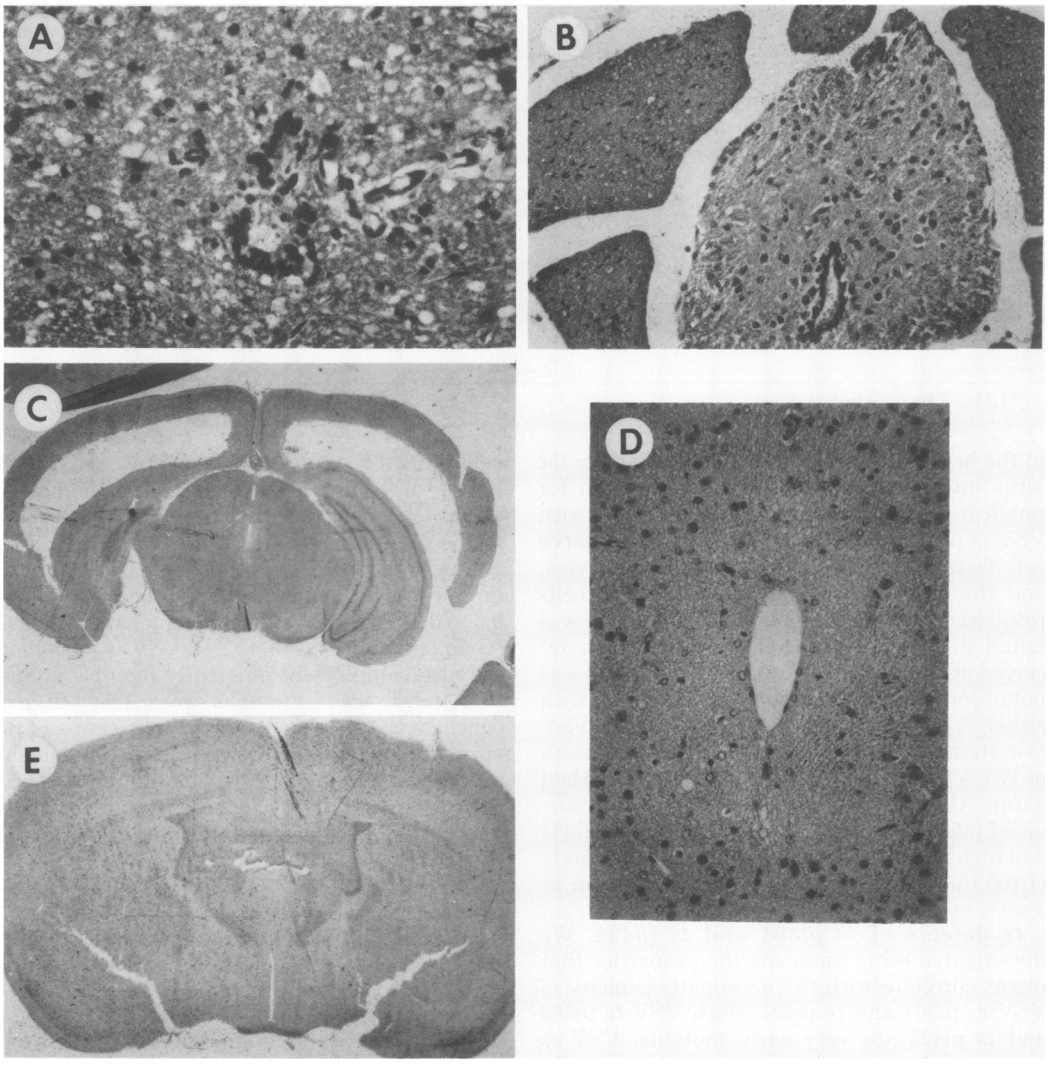

FIG. 1. *Histopathological lesions in mice infected with VSV. Mice were injected intracerebrally with WT VSV (100 PFU per animal) or with VSV ts pi364 (5×10^5 PFU per animal). Sections of brain and spinal cord tissue from animals sacrificed at various times after infection were stained with hematoxylin and eosin. (A) Neuronal necrosis and damage to ependymal cells of the central canal in the sacral spinal cord after infection with WT VSV. (B) Sacral spinal cord of mock-infected control mouse. (C) Massive hydrocephalus 4 weeks after infection with ts pi364. (D) Aqueductal stenosis, with aqueduct denuded of ependyma 4 weeks after infection with ts pi364. (E) Normal cerebral architecture of mock-infected control animal shown for comparison with (C) above.*

TABLE 2. *Neuropathology in the central nervous system of mice infected with WT VSV or VSV ts pi mutants*

Pathological lesion	Severity of morphological changes resulting from infection with[a]:				
	WT VSV	*ts* pi364	*ts* pi421	*ts*$^+$ pi364 R1	*ts* G11
Leptomeningitis	3-4+	3+	3+	3-4+	1+[b]
Encephalitis and myelitis	3-4+	0	2+	2+	0
Nerve cell necrosis	2-4+	0	2+	2+	0
Ependymal cell necrosis[c]	2-4+	2-3+	3+	3+	0
Hydrocephalus	0	3-4+[d]	0	0	0

[a] Changes graded on a scale of 0 to 4+, with 0 indicating no morphological changes and 4+ indicating severe morphological changes.

[b] Found only in animals sacrificed later than 1 to 2 months after infection.

[c] Ependymal cell necrosis was primarily in the spinal cord after infection with WT VSV, *ts* pi421, and *ts*$^+$ pi364 R1, but was limited to the ventricles in the brain after infection with *ts* pi364 (Barmada et al., submitted for publication).

[d] Hydrocephalus was apparent by 4 weeks after infection in >90% of animals infected with >100 PFU of *ts* pi364.

population is presented below.

In mice infected with VSV *ts* pi364 (Fig. 2A, open circles), initial virus replication was similar to that seen with WT VSV. However, virus replication plateaued between 30 and 72 h and then rapidly declined. By 5 days postinfection, virus was no longer detectable in tissue homogenates, although *ts* virus was still recovered up to 10 days postinfection by cocultivating brain tissue with Vero or BHK-21 cells (data not shown). The in vivo growth curve of VSV *ts* G11 (Fig. 2A, closed triangles) agreed with previous observations by others (5, 6, 19) and with the results of the cut-off temperature experiment. Virus replication was very limited, and infectivity was undetectable in 10% homogenates by 96 h postinfection. However, cocultivation of brain tissue with Vero or BHK-21 cells revealed the presence of *ts* virus until 9 days postinfection.

The replication of WT VSV and *ts* pi421 in the spinal cord (Fig. 2B) correlated well with the results obtained in the brain; the maximum concentration of virus in the brain and spinal cord was essentially the same, and virus was not detected in the spinal cord until replication in the brain was well under way. The delay in replication of *ts* pi421 in the brain (Fig. 2A) was reflected in a 24-h delay in the appearance of virus in the spinal cord. The growth curve of *ts* pi364 in the spinal cord of infected mice (Fig. 2B, open circles) was distinctly different from that of either WT VSV or *ts* pi421. A slow, gradual rise in virus concentration occurred between 30 and 96 h after infection. However, by 6 days postinfection, virus had declined to undetectable levels. No virus replication was detected in the spinal cord of mice infected with *ts* G11 (Fig. 2B, closed triangles), in agreement with previous results (19). The results shown in Fig. 2 are therefore consistent with the pathological findings (Fig. 1; Barmada et al., submitted for publication) and also with previous suggestions that the course of clinical disease in mice infected with VSV correlates with the extent of viral replication in the spinal cord (19).

Virus antigens in infected mice. In view of the dramatic difference in the histopathology after infection with these VSV *ts* pi mutants, we also studied the distribution and spread of virus antigens during infection. The objective was to determine whether VSV *ts* pi364 was excluded from neurons or whether neurons were infected without cytopathology. Frozen sections prepared from the brains, brainstems, and cervical spinal cords of mice sacrificed at various times during the growth curve experiments shown in Fig. 2 were processed by using indirect fluorescent-antibody techniques. Companion sections were also stained with hematoxylin and eosin and examined for histopathological lesions. Immunofluorescent staining of brain sections from mice undergoing infection with WT VSV revealed viral antigen throughout the neuropil by 48 h after infection. There was intense fluorescence in the meninges and in the ependymal cells lining the ventricular cavities of the brain (Fig. 3A) as well as in the central gray nuclei (thalmus) and throughout the brainstem and cervical spinal cord. Bright fluorescence was observed in virtually every tissue element in brain sections prepared from moribund animals 48 to 72 h after infection.

There was a similar distribution of virus antigen in the brains and cervical spinal cords of mice infected with VSV *ts* pi421. Antigen was first detected at 48 h after infection in the meninges; by 64 h, intense staining of the ependymal lining of the ventricles (Fig. 3B) and leptomininges (Fig. 3C) was evident, with foci of viral infection in the neuropil. By 5 to 6 days, many foci of virus antigen were scattered throughout the brains (Fig. 3D) and cervical spinal cords of

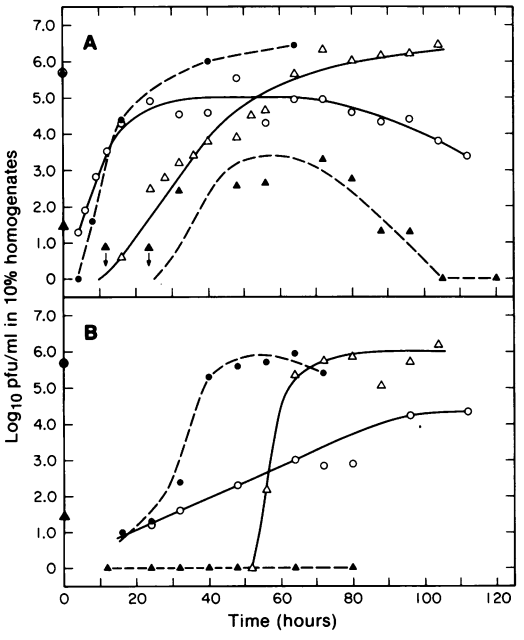

FIG. 2. *Replication of VSV in brain and spinal cord of infected mice. Mice were inoculated intracerebrally with WT VSV (100 PFU), ts pi364 (5.0 × 10^5 PFU), ts G11 (5 × 10^5 PFU), or ts pi421 (100 PFU) and sacrificed at various times after infection. 10% homogenates were prepared from brains (A) or spinal cords (B), and virus was assayed on CE cells at 34°C. Each point represents infectivity in pooled tissue from two mice. Symbols: ●, WT VSV; ○, ts pi364; ▲, ts G11; △, ts pi421.*

remaining moribund animals. These observations correlate very well with the histopathology of infection seen in mice infected with WT VSV or *ts* pi421.

Animals infected with VSV *ts* pi364 showed no encephalitis, a less severe leptomeningitis, more severe damage to the ependymal cells of the lateral ventricles, and little or no destruction of nerve cells. The fluorescent-antibody stainings observed in these mice were consistent with these pathological findings. As early as 24 h postinfection, viral antigen was evident in the lateral ventricles and leptomeninges. The intensity of staining was at its maximum at 72 h after infection, but virus antigen was confined to the leptomeninges (Fig. 3E) and the ependyma of the lateral ventricles (Fig. 3F). A much lower level of virus antigen was observed in the ependymal lining of the third ventricle, and foci of virus antigen were never seen in the neuropil. The brainstem and cervical spinal cord also lacked detectable virus antigens at any time after infection, a finding consistent with the absence of spinal cord pathology in animals infected with VSV *ts* pi364.

The nature of the histopathological lesions and the restriction of viral antigens in animals infected with *ts* pi364 suggests that this virus mutant, in contrast to WT VSV and *ts* pi421, is unable to infect and replicate in neurons. VSV *ts* pi364 may therefore be a mutant with an altered cell tropism in vivo and a useful tool in further studies of the virus-specific factors which influence neuropathogenicity in vivo. However, several other possible explanations for the difference in neurovirulence of these two *ts* pi mutants also were investigated.

Interferon response in infected mice. It was possible that nonlethal VSV mutants such as *ts* pi364 and *ts* G11 induced high levels of either local or circulating interferon, whereas WT VSV and the virulent *ts* pi421 mutant did not induce interferon. Therefore, selected brain homogenate samples, obtained from the growth curve experiments (Fig. 2), were assayed for interferon. Interferon was undetectable in all but two samples. A brain homogenate from mice infected with VSV *ts* G11 had an interferon titer of 1:16 at 48 h postinfection, whereas 32 U of interferon were found in a brain homogenate prepared from mice 40 h after infection with WT VSV. Therefore, the presence of interferon in the brain does not correlate with neurovirulence.

Figure 4 shows that substantial amounts of interferon were present in the sera of mice infected with all four virus types. Although the highest levels of interferon were reached early in infection, a second peak of interferon production occurred later in infection with both *ts* pi364 and 421 (Fig. 4C and 4D). This biphasic curve has also been demonstrated with other viruses (33). One explanation of the second peak of serum interferon might be that a different cell population was exposed to virus later in infection and responded with interferon production. Both early and late samples appeared to contain authentic interferon since the antiviral factor was stable during incubation at pH 2 and was able to protect mouse L cell cultures from infection with Mengo virus (data not shown). However, the presence of a low level of antiviral antibody in the later serum samples was not rigorously excluded.

Characterization of the virus population in infected mice. Another explanation for the difference in neurovirulence of *ts* pi364 and *ts* pi421 may be the accumulation of non-*ts* revertants during infection with *ts* pi421; these revertants may cause the encephalitis and death in the infected mice. Virus was isolated from brain and spinal cord homogenates obtained during two virus growth curve experiments with *ts* pi364 and *ts* pi421 (Table 3). The infectivity data from the two experiments agreed very well and had

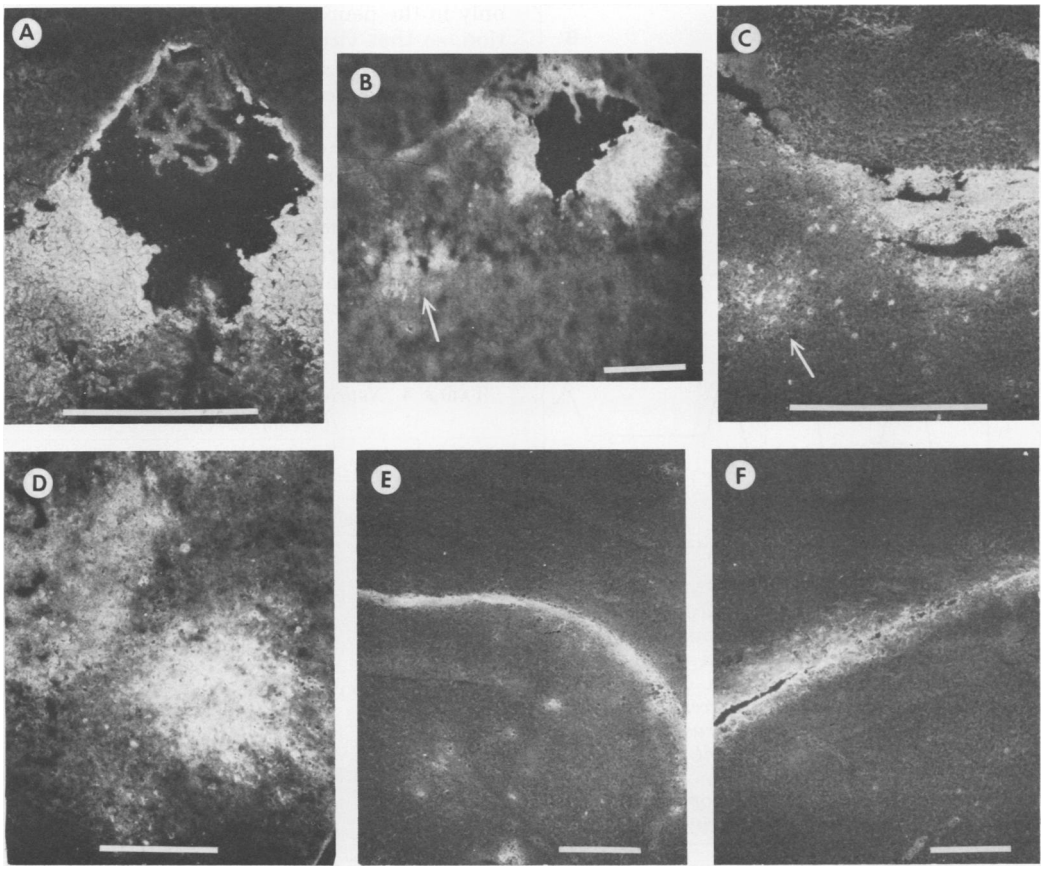

FIG. 3. *Distribution of viral antigens in the brains of VSV-infected mice. Frozen sections (4 to 8 μm) were prepared from brains harvested at various times after intracerebral infection of mice with 100 PFU of WT VSV (A), 100 PFU of ts pi421 (B, C, and D), or 5 × 10^5 PFU of ts pi364 (E and F). The sections were processed for indirect immunofluorescence as described in the text. WT VSV antigen was seen in the ependymal cells of the third ventricle and in surrounding neuropil at 36 h after infection (A). VSV ts pi421 antigen was found in the third ventricle (B) and leptomeninges (C) at 64 h after infection. Note foci of viral antigen in neuropil in (B) and (C) (arrows). Many adjacent foci of viral antigen were seen throughout the neuropil by 5 to 6 days after infection with ts pi421 (D). VSV ts pi364 viral antigen at 72 h after infection was confined to the leptomeninges (E) and ependyma of the lateral ventricles (F). Magnification bar denotes 0.1 mm.*

been pooled to generate Fig. 2.

In experiment 1, only 7 out of 19 *ts* pi421 clones isolated from the brain and spinal cord at 72 h after infection were temperature sensitive. However, when the analysis was repeated using 72-, 88-, and 104-h samples from experiment 2, all of the virus population in the brain, and the majority in the spinal cord, remained temperature sensitive. Nevertheless, the concentration of virus in the brain, the time of death of infected animals, the histopathological lesions, and the distribution of viral antigens were identical in both experiments. The reason for the variation between experiments 1 and 2 is unclear and may be due to individual differences in the outbred mice.

Analysis of virus isolated from animals infected with *ts* pi364 showed that 100% of the virus population in the brain was temperature sensitive at 48 and 96 h after infection. A significant proportion (35%) of the virus population present in the spinal cord of *ts* pi364-infected mice was not temperature sensitive at 72 h after infection. However, all of the mice were apparently healthy at the time of sacrifice, and the pathological lesions and pattern of immunofluorescence were typical for infection with *ts* pi364.

These results indicate that non-*ts* revertants occasionally arise during infection with either of the *ts* pi viruses and that accumulation of these revertants is probably not the major determinant in the difference in neurovirulence between *ts* pi364 and *ts* pi421.

It was also possible that a change occurred

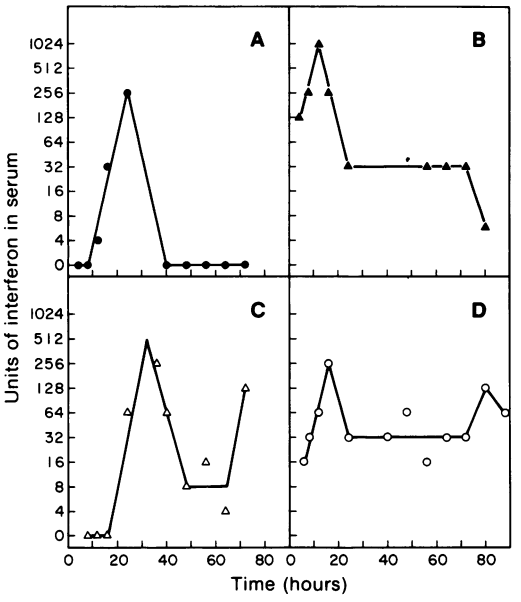

FIG. 4. *Serum interferon in mice infected with VSV. Serum interferon was determined at various times after intracerebral inoculation of mice with WT VSV (A), ts G11 (B), ts pi421 (C), or ts pi364 (C). Samples (24 to 48 h) in (A) and (C) were pretreated at pH 2 to eliminate the infectious virus present; other sera were assayed without treatment.*

TABLE 3. *Screening of virus population for temperature sensitivity*

Virus	Experiment no.	Time after infection (h)	Source of clones[a]	No. of *ts* clones isolated (%)[b]
ts pi421	1	72	Brain	4/10 (40)
			Spinal cord	3/9 (33)
	2	72	Brain	7/7 (100)
			Spinal cord	20/20 (100)
		88	Brain	20/20 (100)
			Spinal cord	15/20 (75)
		104	Brain	20/20 (100)
			Spinal cord	19/20 (95)
ts pi364	1	96	Spinal cord	24/25 (96)
	2	48	Brain	20/20 (100)
		72	Spinal cord	13/20 (65)
		96	Brain	16/16 (100)

[a] Plaques picked from assay plates of 10% tissue homogenates.

[b] An efficiency of yield in screening experiments of 10^{-3} or less was used as the criterion of temperature sensitivity. The yield efficiency of WT VSV was 0.5 to 1.0 in different experiments.

only in the neurovirulence of the virus population, so that virulent but still *ts* virus evolved late in infection and was responsible for death of the animals infected with *ts* pi421. Several of the *ts* virus isolates from experiment 2 were therefore chosen at random and tested for virulence in mice. The efficiency of yield at 39.5°C of the virus clones tested ranged from $<10^{-7}$ to 1.5×10^{-6}. Table 4 shows that both *ts* pi364-derived and ts pi421-derived virus clones were slightly more lethal in mice than the corresponding parental *ts* pi virus. The *ts* pi421-derived clones, however, closely resembled the parental *ts* pi421

TABLE 4. *Neurovirulence of virus clones isolated from infected mice*

Virus clone[a]	LD_{50}[b] (PFU per mouse)	Day of death	Clinical observations
4-B42	<10	3-7	Ruffled fur, spasms, seizures
4-B48	<10	3-5	Ruffled fur, spasms, seizures
4-B59	<10	3-4	Ruffled fur, spasms, seizures
4-SC46	10^2	4-9	Hlp[c] 5 days p.i.[c] in 4/8 surviving mice
4-SC54	<10	3-4	Ruffled fur, spasms, seizures
4-SC59	<10	2-4	Ruffled fur, spasms, seizures
3-B4	$>10^{5d}$	3[e]	
3-B12	$>10^5$	9	Hlp 6 days p.i. in 2/15 surviving mice
3-B18	$>10^5$	5-9	Hlp 5 days p.i. in 3/13 surviving mice
3-B21	$>10^5$	5	Hlp 5 days p.i. in 3/15 surviving mice
3-B27	$>10^5$	6-8	Hlp 5 days p.i. in 7/13 surviving mice
3-B33	$>10^5$	5-6	Hlp 5 days p.i. in 1/14 surviving mice
WT	<1	2-3	Ruffled fur, spasms, seizures
ts pi364	$>10^7$	5-9[e]	Hlp in 10 to 15% of mice
ts pi421	20	3-5	Ruffled fur, spasms, seizures

[a] Virus clones designated 3- were obtained from *ts* pi364-infected mice; virus clones designated 4- were obtained from *ts* pi421-infected mice; B-numbered clones were picked from brain homogenates; and SC-numbered clones were from spinal cord samples.

[b] LD_{50}, The 50% lethal dose.

[c] Hlp, Hind leg paralysis; p.i., postinfection.

[d] 10^5 PFU per mouse was the highest dose tested.

[e] For all 3- virus clones and for *ts* pi364, day of death figure indicates days on which an occasional mouse died; most animals remained clinically healthy.

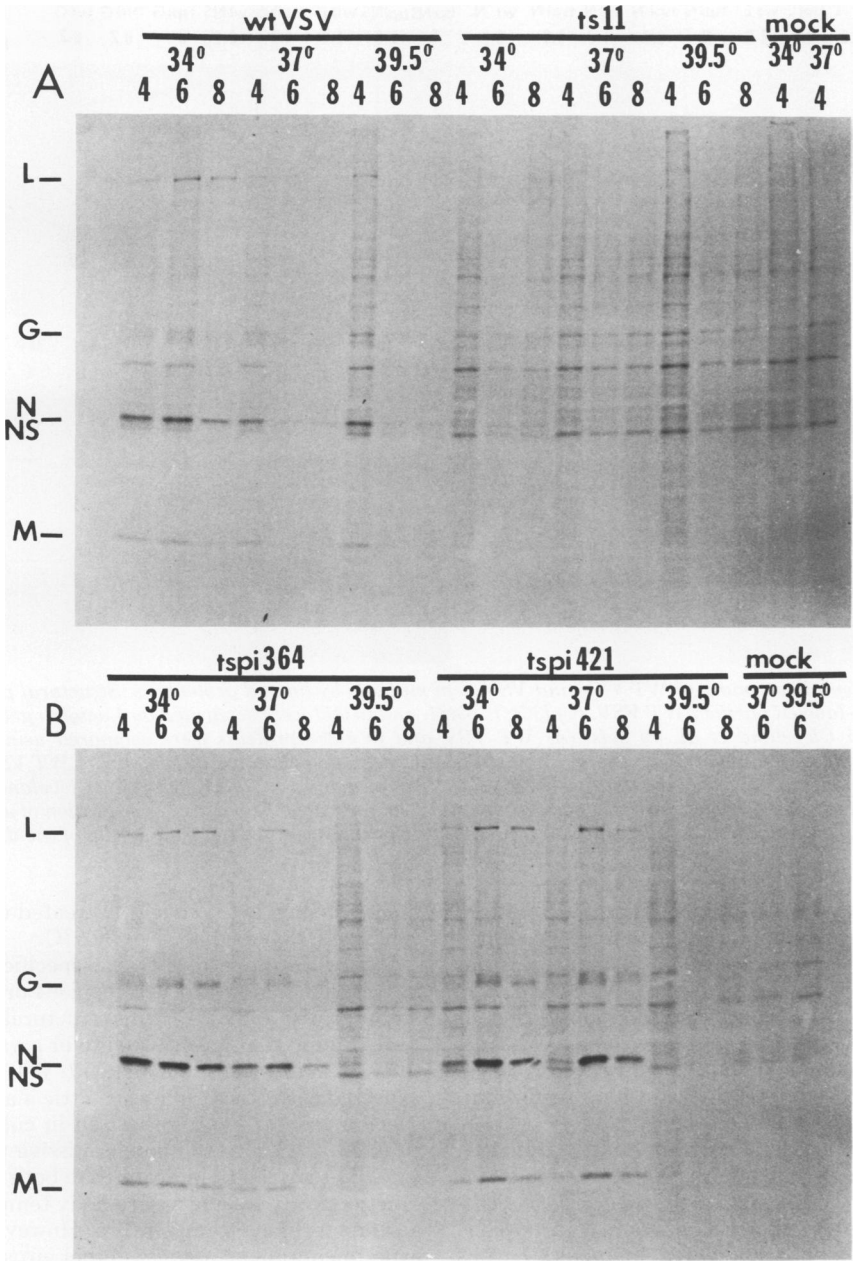

FIG. 5. *Virus-specific protein synthesis in L cells infected with WT VSV or VSV ts pi mutants. Mouse L cell monolayers were infected with WT VSV, ts G11, ts pi364, or ts pi421 (10 PFU per cell) for 1 h at 4°C, washed, fed, and incubated at 34, 37, or 39.5°C in the presence of 5 μg of actinomycin D per ml. Virus-specific proteins were labeled with [^{3}H]leucine for 30 min at the times indicated, after which monolayers were solubilized directly in the Laemmli sample buffer. Equal volumes (25 μl) of cell extract were applied to 10% Laemmli gels, and electrophoresis was continued for 40 min after the bromophenol blue marker dye migrated off the gel. An autoradiogram of the dried gel is shown.*

virus, rather than WT VSV, in the time of death of infected mice. This suggests that large changes in the neurovirulence of the virus population did not occur. One virus clone, 4-SC64, was less lethal than parental *ts* pi421 and produced a clinical disease syndrome similar to that of *ts* pi364. Further studies of this virus clone are in progress. All of the virus clones derived from

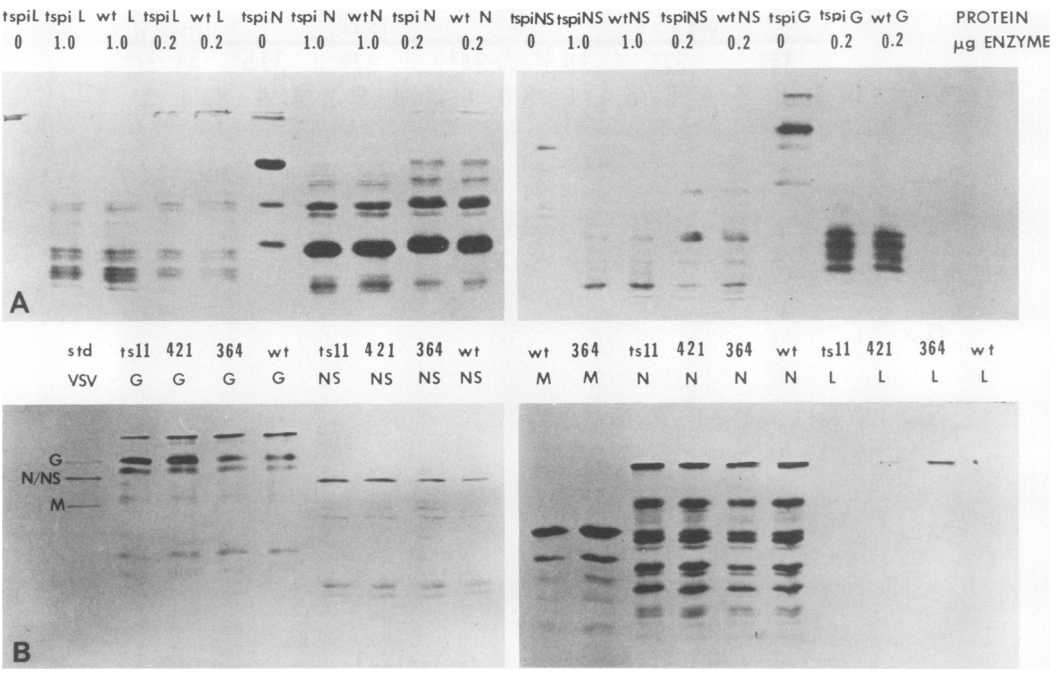

FIG. 6. *Peptide mapping of WT VSV and VSV ts pi mutants by limited proteolysis. Structural proteins of [^{3}H]leucine-labeled purified WT VSV, ts pi364, ts pi421, and ts G11 were separated on Laemmli gels (15) and then run on Cleveland et al. (3) gels. (A) WT VSV and ts pi364 proteins were compared using various concentrations of S. aureus V-8 protease. Identical results were also obtained by comparing WT VSV with ts pi421 or comparing the two ts pi mutants directly. (B) Viral proteins were analyzed by the Cleveland et al. (3) method using 20 μg of chymotrypsin per sample well. VSV std indicates the position of migration of undigested viral proteins. Note little if any digestion of L protein by chymotrypsin. An autoradiogram of the dried gel is shown.*

the brain of *ts* pi364-infected animals produced hind leg paralysis of infected animals at a much lower dose than parental *ts* pi364, indicating that some neuroadaptation might have occurred in infected animals. These results support previous suggestions that the altered virulence of some VSV *ts* mutants is not dependent on the *ts* mutations per se and that another "neuropathogenicity" locus may also exist (20).

In vitro studies with *ts* pi364 and 421. Since further study of virus-cell interactions was difficult to pursue in vivo, the synthesis of virus-specific proteins during infection with *ts* pi364 and *ts* pi421 was compared in mouse L cells incubated at various temperatures. As shown in Fig. 5A, all of the virus-specific proteins were present by 4 h after infection of L cells with WT VSV regardless of the incubation temperature. Even with WT VSV, virus protein synthesis was more efficient at 34°C than at 37°C or 39.5°C. In contrast, in L cells infected with *ts* G11, virus-specific proteins were barely detectable at 6 or 8 h after infection at 34°C (Fig. 6A); as expected from the cut-off temperature experiments, only host-specific proteins, identical to those seen in mock-infected actinomycin D-treated control L cells, were detected at 37 or 39.5°C.

Figure 5B compares the virus-specific proteins in L cells infected with VSV *ts* pi364 or *ts* pi421. At both 34°C and 37°C, virus structural proteins were produced at levels equal to or greater than those seen in L cells infected with WT virus. As expected with RNA$^-$ mutants, little if any virus-specific protein was synthesized in cultures incubated at 39.5°C, the nonpermissive temperature. These results indicate that both mutants function fairly well at mouse body temperature, a semipermissive temperature. However, these experiments do not assess whether virus proteins are properly compartmentalized or modified posttranslationally in infected cells at semipermissive temperatures. In addition, infection of mouse L cells may not accurately reflect the events in specialized cells of the nervous system; further analysis of these *ts* pi mutants in explanted mouse embryo neuron cultures (7, 29) is in progress.

We have also investigated the proteins found in purified virus. There was no difference in the migration of any of the five viral structural pro-

teins when WT VSV, *ts* pi364, *ts* pi421, and *ts* G11 were compared on 10% polyacrylamide slab gels prepared by the method of Laemmli (15). Furthermore, each of the virion proteins of both *ts* pi364 and *ts* pi421 appeared to be very similar to the corresponding protein of WT VSV using the Cleveland et al. (3) technique of limited proteolysis. Figure 6A shows a comparison of WT VSV and *ts* pi364 proteins using *S. aureus* V-8 protease; comparison of WT VSV with *ts* pi421 or direct comparison of the two *ts* pi mutants yielded identical results (data not shown). Digestion of the proteins of all four viruses with chymotrypsin is shown in Fig. 6B. Although little if any digestion of L protein was observed, no differences were evident in any of the other structural components of these viruses. However, this method would only detect major differences between proteins; classical two-dimensional tryptic peptide fingerprinting is currently in progress to detect more subtle differences between the virion proteins of *ts* pi364 and *ts* pi421. It is possible that a non-*ts* lesion in another protein, such as the G surface glycoprotein, is responsible for the difference in target cell tropism we have observed.

DISCUSSION

The results presented above indicate that VSV *ts* pi364 has an altered target cell range in vivo. As far as we are aware, this is the first report of an in vivo target cell-specific mutant of VSV. Unlike WT VSV and VSV *ts* pi421, which infect both neurons and ependymal cells after intracerebral infection of mice, VSV *ts* pi364 does not infect neurons. In animals infected with *ts* pi364, viral antigens and histopathological lesions are limited almost exclusively to the leptomeninges and the ependyma of the lateral ventricles. Infected mice survive and develop hydrocephalus as a delayed sequela of infection.

The VSV *ts* pi mutants were both isolated from a persistently infected L_{VSV} culture initiated with WT VSV (36). Both *ts* pi364 and *ts* pi421 are RNA^- *ts* mutants, produce small plaques in CE cells, and have identical cut-off temperatures as well as low reversion frequencies both in vitro and in vivo. The only phenotypic difference thus far detected between *ts* pi364 and *ts* pi421 is the difference in neurovirulence in mice.

Genotypically, the *ts* defect of VSV *ts* pi364 maps in complementation group I (L protein), whereas the *ts* mutation of *ts* pi421 appears to be in the N protein (group IV). Our data therefore confirm previous conclusions by others (20, 30) that there is no apparent correlation between the complementation group and the degree of neuropathogenicity of a given VSV *ts* mutant. VSV *ts* pi364 and *ts* G11 are two group I mutants, yet *ts* pi364 produces an abnormal pathological disease in infected mice, whereas *ts* G11 produces no disease at all. Furthermore, unlike our group IV mutant, *ts* pi421, other group IV mutants such as *ts* G41 produce no pathological lesions in infected Swiss mice (19). In addition, Rabinowitz et al. (20) found that the temperature sensitivity of both *ts* G31 and *ts* G22 is dissociable from their characteristic neuropathogenicity in mice, suggesting the existence of a separate neuropathogenicity locus. Similar results were also obtained with foot-and-mouth disease virus (21). Therefore, it is unlikely that the *ts* defects per se in VSV *ts* pi364 and *ts* pi421 are responsible for the difference in target cell tropism observed in vivo.

A similar difference in virulence and target cell tropism exists between reovirus types 1 and 3. Newborn mice infected with reovirus type 3 develop a fatal acute encephalitis accompanied by neuronal destruction without damage to ependymal cells. Animals infected with reovirus type 1 survive no neuronal necrosis but show ependymal cell damage and delayed hydrocephalus (34). Genetic recombination techniques were used to determine that the S1 double-stranded RNA genome segment is the major determinant of neurovirulence. Since this genome segment codes for the reovirus hemagglutinin, an outer capsid protein, Weiner et al. (35) postulated that the difference in cell tropism between reovirus types 1 and 3 is due to specific interaction of the type-specific hemagglutinin with receptors on the surface of ependymal cells or neuronal cells, respectively. Since both the L and N proteins of VSV are internal structural components of the ribonucleoprotein core and are involved in transcription and replication of viral RNA, direct involvement of either of these proteins in the initiation of infection is unlikely. Although we have not detected any differences in the G proteins of WT VSV, *ts* pi364, and *ts* pi421, and all of the viruses are neutralized to the same extent by anti-WT VSV antiserum, a minor non-*ts* change in the G protein of *ts* pi364 remains a possibility.

The studies with reovirus also suggested that "secondary" virulence genes exist which produce their effects through control of viral replication in vivo rather than by determination of virus-host interactions at the cell surface (35). This hypothesis is supported by recent experiments with influenza virus (23–25) which showed that virulence for chickens of fowl influenza type A recombinants was dependent on the polypeptide composition of the viral RNA polymerase. All of the nonpathogenic recombinants derived from two virulent fowl influenza viruses had a com-

bination of both parental types in the four viral proteins (pol 1, pol 2, ptra, and NP) which together form the RNA polymerase complex (23). Efficient virus replication in appropriate target cells, leading to death of the infected animal, may therefore depend on proper interaction of the virus-specific polymerase complex with some host cell factor(s) peculiar to that cell type. Conversely, mixing of gene segments coding for polymerase proteins from two avirulent influenza viruses may result in a highly virulent recombinant (26).

VSV *ts* pi364 may therefore have a second non-*ts* mutation in one of the proteins (L, N, or NS) of the polymerase complex which can function in vivo in ependymal cells but not in neurons. This type of host range mutation would account for the restricted target cell specificity of *ts* pi364. Mutants of VSV with an altered host range in vitro have been described previously (17, 27, 30, 32). Most of the host range mutants unable to grow in human cells (27) were also temperature sensitive in permissive CE cells; both RNA^+ and RNA^- mutants were found. Some temperature-dependent host range mutants had a virion transcriptase which was defective in an in vitro reaction system (31), and the defect was probably in the L protein (32). Experiments to test this hypothesis in explanted mouse embryo neuron cultures in vitro are in progress.

Finally, it is also possible that *ts* pi421 and *ts* pi364 produce different types of defective interfering particles in vivo and that this results in differential progression of infection. Although we consider a significant role for defective interfering particles unlikely in explaining the inability of *ts* pi364 to infect neurons, experiments to rule out this possibility, using in vitro neuron cultures, are also in progress.

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