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Comparison of Central Nervous System Disease Produced by Wild-Type and Temperature-Sensitive Mutants of Vesicular Stomatitis Virus

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The pathogenicity of infection produced following intracerebral (i.c.) inoculation of wild-type vesicular stomatitis virus (VSV) or temperature-sensitive *(ts)* mutants of VSV was compared. *ts* mutants used were *ts* 31 (VSV complementation group II) and *ts* 41 (VSV complementation group IV). The i.c. injection of wild-type VSV in weanling Swiss mice produced a rapidly fatal encephalitis with death of mice in 2 to 3 days. Histopathologically, such mice exhibited minimal changes of encephalitis on light microscopy. In contrast to the highly virulent, rapidly fatal central nervous system (CNS) infection seen after i.c. inoculation of wild-type VSV, infection with *ts* 31 VSV produced a more slowly progressive CNS infection characterized by hind limb paralysis and death 6 to 9 days after infection. Histopathologically, CNS infection with *ts* 31 is associated with previously unreported extensive spongiform changes in the gray matter of the spinal cord. The inoculation of *ts* 41 i.c., on the other hand, did not result in either clinical illness or histopathological changes in the spinal cords or brains of infected mice. The absence of clinical and histopathological lesions following i.c. infection of *ts* 41 VSV suggests that the capacity to alter the pathogenesis of VSV CNS infection may be a function of only certain *ts* mutants of VSV.

Considerable investigative effort has been directed at defining mechanisms that explain the nature of persistent virus infection. A number of the studies have used carrier cell cultures as models of persistent virus infection (8, 13). Many viruses, even some considered highly cytocidal, can establish infection of cell cultures which result in persistent virus multiplication while the cell culture continues to survive and grow (13). Such cell cultures which continue to grow and survive after establishment of viral infection are defined as carrier cultures. One of the more important mechanisms by which persistently infected carrier cultures are maintained is through the selection or evolution of temperature-sensitive *(ts)* mutants in cell cultures (8). In spite of the extensive body of data concerning the role *ts* mutants play in vitro, little is known of their role, if any, in disease production in vivo.

Recently, Holland and Villarreal (4) described a carrier culture model involving vesicular stomatitis virus (VSV). VSV infection of cells is usually highly cytocidal, with death of the cells in less than 24 h. But, by infecting cells with both defective-interfering (DI) particles of *ts* 31 and standard *ts* 31 VSV particles, a chronic carrier culture was established. Furthermore, DI particles generated during the establishment of chronic *ts* 31 VSV infection in vitro, mixed even with highly virulent wild-type (wt) VSV, could now be used to establish carrier cultures. Because of these studies, we considered it worthwhile to explore the possibility that certain *ts* mutants of VSV could alter the usually highly virulent central nervous system (CNS) disease produced by wt VSV infection of mice into a more slowly progressive infection. The purpose of these studies was, therefore, to describe the in vivo CNS disease produced after intracerebral (i.c.) inoculation of wt or certain *ts* mutants of VSV.

Our results suggest that in contrast to wt VSV, CNS infection produced after i.c. inoculation of *ts* 31 is slowly progressive and associated with previously unreported spongiform changes in the gray matter of the spinal cord. In contrast, i.c. infection with *ts* 41 results in neither clinical signs nor histological lesions in the CNS.

MATERIALS AND METHODS

Animals. Outbred Swiss mice of both sexes, 3 to 4 weeks of age, were purchased from Scientific Products (Arlington Heights, Ill.). All mice were provided with food and water ad libitum.

Cell culture lines. BHK-21 cells originally were obtained from International Scientific Industries (Cary, Ill.). BHK-21 cells were grown to confluence in minimal essential medium with Earle salts supplemented with 7% fetal calf serum (virus-screened, GIBCO), 10% (wt/vol) tryptose phosphate, 2 mM L-glutamine, 1% (vol/vol) nonessential amino acids, 1% (vol/vol) minimal essential medium vitamins, and 100 U of penicillin, 100 μg of streptomycin, and 2.5 μg of amphotericin B. Hereafter this medium will be referred to as BHK-21 growth medium. BHK-21 maintenance medium is the same as the above formula except for the fact that 4% fetal calf serum is used.

Viruses. (i) wt VSV. Indiana strain VSV was originally obtained from the American Type Culture Collection. Stock virus was prepared in 75-cm^2 flasks (Falcon Plastics) containing confluent monolayers of BHK-21 cells. BHK-21 cell monolayers were infected with dilute passage wt VSV (~0.1 plaque-forming units [PFU]/cell). This multiplicity of infection results in production principally, if not exclusively, of standard virions (12). Virus was allowed to adsorb for 60 min at 37 C in a 5% CO_2 atmosphere. Infected cells were then supplemented with maintenance medium and cultured in a 5% CO_2 environment for 18 to 22 h at 37 C. After culture, supernatant fluids were harvested and centrifuged in a Sorvall RC-2B refrigerated centrifuge at 5,000 $\times$ *g* to remove cell debris. VSV prepared in this manner titered 5 $\times$ 10^8 PFU/ml in BHK-21 cells. The VSV was then plaque purified twice in BHK-21 cells. Large quantities of stock, doubly cloned wt VSV were then prepared by infecting 32-ounce (about 960-ml) prescription bottles containing confluent monolayers of BHK-21 cells with dilute passage (0.01 to 0.1 PFU/cell) doubly cloned wt VSV. Virus-infected cells were grown and supernatant fluids were harvested as outlined above. Doubly cloned VSV titered 2 $\times$ 10^9 PFU/ml in BHK-21 cells.

(ii) *ts* mutants. *ts* 31 and *ts* 41 were generously provided by M. E. Reichmann (University of Illinois, Urbana). These *ts* mutants have previously been extensively described by Reichmann and others (9, 11). Each of these mutants was plaque purified in Reichmann's laboratory and subsequently passaged by infecting cells with 0.01 to 0.1 PFU/cell to minimize production of DI particles of the *ts* mutants. *ts* 31 is a member of complementation group III (11) and *ts* 41 is a member of complementation group IV (11). Initially, 25-cm^2 flasks containing confluent monolayers of BHK-21 cells were infected with dilute passage (~0.1 PFU/cell) *ts* virus. Virus-infected cells were cultured as outlined above, except that BHK-21 cells infected with *ts* 31 and *ts* 41 were grown at 31 C instead of 37 C. Supernatant fluids were harvested 20 to 24 h after infection with *ts* 41 and 48 h after infection with *ts* 31. Virus-containing fluids were clarified by centrifugation at 5,000 $\times$ *g* in an RC-2B refrigerated centrifuge and then passaged at 31 C in 75-cm flasks of BHK-21 cells so as to produce a large pool of *ts* mutant stock virus. Again, dilute passage (~0.01 to 0.1 PFU/cell) *ts* mutant virus was used to infect BHK-21 cells to reduce, if not eliminate, the production of DI particles. This was considered important in pathogenesis studies, since we wanted to reduce the possibility that i.c. infection of mice was being produced by *ts* virus preparations containing variable amounts of DI particles and standard virus. Yields of *ts* mutants, as determined by plaquing in BHK-21 cells at 31 C, were: (i) *ts* 31, 4.7 $\times$ 10^7 PFU/ml; (ii) *ts* 41, 5.55 $\times$ 10^8 PFU/ml.

Plaque assay. BHK-21 cells (5.0 $\times$ 10^5 to 7.5 $\times$ 10^5/ml) 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. Confluent monolayers developed in 24 to 36 h and were infected by adding 0.1-ml $\log_{10}$ dilutions of freshly thawed virus stock to each series of wells. Virus was allowed to adsorb for 60 min at either 31 or 37 C in a 5% CO_2 atmosphere, and wells were overlayed with 2 ml of Eagle basal medium supplemented with 5% fetal calf serum, 5% (wt/vol) tryptose phosphate, 2 mM L-glutamine, 100 U of penicillin, 100 μg of streptomycin, 2.5 μg of amphotericin B, and 0.7% Ionagar (Colab Labs, Chicago). Virus samples were then grown at 31 C for fluids containing *ts* mutants and 37 C for fluids containing wt VSV. After incubation for 24 h at the appropriate temperatures, cultures were counterstained with a second overlay containing neutral red (1:9,000). All samples were plaqued in triplicate, and plaques were counted 18 h after the second overlay by inverting the plates over an X-ray viewbox.

Ultraviolet inactivation of VSV. The *ts* 31 VSV was placed in a 100-mm sterile tissue culture dish and irradiated by a Sylvania germicidal lamp, G15T8, at a distance of 8 cm for 10 min with constant swirling. After ultraviolet (UV) inactivation, aliquots of *ts* 31 were either plaqued at 31 C in BHK-21 cells or grown in 25-cm^2 flasks of BHK-21 cells in an attempt to determine whether any residual virus remained. No residual infectious virus was detected by either method.

Hyperimmune serum. Doubly cloned wt VSV was used to raise antibody in rabbits. Before injection, three rabbits were bled from the ear, and the sera were separated and then stored at −20 C. These control sera tested individually were shown to contain <1:10 titer of neutralizing antibody to VSV. These rabbits were then injected with 10^8 PFU of wt VSV intravenously via the ear veins. VSV-injected rabbits were boosted intravenously weekly for 5 consecutive weeks with 10^8 PFU of VSV. Two weeks after the last inoculation of VSV, rabbits were exsanguinated and the sera were pooled and titered for neutralizing antibody by standard plaque reduction. The pooled serum was shown to have a neutralizing antibody titer of 1:64,000.

Histopathological studies. Mice were anesthetized by intraperitoneal injection of 0.02 ml of Diabutal (50 mg/ml) and sacrificed by total body perfusion with cold (4 C) 3% glutaraldehyde in phosphate buffer (pH 7.4). Tissue blocks from brain and spinal cord were minced and postfixed for 60 min in 1% osmic acid in phosphate buffer (pH 7.4). After dehydration and Epon embedding, 1-μm sections were cut and stained with toluidine blue for light-microscopy studies. Selected fields were trimmed for ultrathin sectioning and studied in a Philips 200 electron microscope. Mice infected with wt VSV were sacri-

ficed for histological examination 24, 48, and 72 h after i.c. inoculation. In these experiments, mice could not be examined after 72 h because they all succumbed to infection. Mice infected with *ts* mutants of VSV were sacrificed and the brain and spinal cord were examined daily for 7 days.

RESULTS

Initial experiments compared the susceptibility of weanling mice (Swiss, 3 to 4 weeks old) to infection with wt, *ts* 31, and *ts* 41 VSV. Groups of 6 to 10 mice were injected with $\log_{10}$ dilutions of each virus preparation i.c., and the 50% mean lethal dose was determined by the method of Reed and Muench (10).

Table 1 compares the amounts of virus required to cause death from infection when the three VSV preparations were administered by the i.c. route. The mean lethal dose for wt VSV was 50 PFU, whereas *ts* 31 required 500 PFU. In contrast, *ts* 41 was avirulent even by the i.c. route. Although not shown, intraperitoneal inoculation of these three VSV preparations did not result in death of the mice, with the exception of occasional deaths in the wt VSV-infected group. Thus, although wt VSV is virulent for mice after i.c. inoculation, it has very limited virulence when administered by a peripheral route of challenge even in doses as large as 10^8 PFU.

Clinically, wt VSV injected i.c. produced a rapidly fatal disease with mice dying 2 to 3 days after infection (Table 2). Mice rarely survived 4 days after wt VSV infection. This clinical picture was consistent and reproducible over a wide dose range (2.5×10^2 to 10^6 PFU of wt VSV i.c.). Mice infected with wt VSV exhibited ruffled fur and lethargy before death. In our experiments, we were impressed by the absence of overt paralysis or convulsions except as a preterminal event (Table 2). In contrast to wt VSV infection, mice injected with 10^4 PFU of *ts* 31 i.c. survived 6 to 9 days, with the majority of mice dying by 7 days after infection. Clinically, all mice infected i.c. with *ts* 31 exhibited marked wasting and hind limb paralysis (Table 2). Mice would sometimes move about the cage, dragging their hind limbs, for 2 to 3 days before death.

To be sure that *ts* 31 virus rather than products of the tissue culture medium or BHK-21 cells or a second unrelated virus was responsible for this slowly progressive CNS virus infection, mice were inoculated with UV-inactivated *ts* 31 virus. UV-inactivated *ts* 31 failed either to plaque at 31 C in BHK-21 cells or to grow in 25-cm^2 flasks of BHK-21 cells. Moreover, mice inoculated i.c. with various dilutions of UV-inactivated *ts* 31 remained clinically well throughout 14 days of observation. Furthermore, mice were infected i.c. with 2×10^6 PFU of *ts* 31 after prior incubation of virus with control or anti-VSV antisera (Table 3). Mice infected i.c. with 2×10^6 PFU of *ts* 31 succumbed to infection between 5 to 9 days after inoculation. Mice infected with *ts* 31 that had been incubated with a 1:50 dilution of normal rabbit serum also succumbed to *ts* 31 between 6 to 9 days. In contrast, mice infected with *ts* 31 incubated before inoculation with a 1:50 dilution of anti-VSV antisera failed completely to exhibit clinical illness or die. Finally, although not depicted, *ts* 31 virus could be recovered from brain and spinal cord 4 to 6 days after i.c. infection in titers ranging from 10^4 to 10^6 $\log_{10}$ PFU per ml of brain or spinal cord. Thus, it is clear that slowly progressive CNS disease seen after i.c. inoculation of *ts* 31 VSV is dependent on the presence of *ts* 31 VSV.

Mice infected i.c. with *ts* 41 (10^5 PFU), on the other hand, remained, in general, clinically well throughout 14 days of observation. A mouse would rarely develop some sluggishness and ruffled fur, but no deaths were ever observed (Table 2). Thus, mice survived i.c. challenge with *ts* 41 and remained well. Although not depicted, challenge of *ts* 41-infected mice 21 days after inoculation with 10^4 PFU of wt VSV resulted in survival of six of eight mice. These results suggest that i.c. inoculation of *ts* 41 probably results in some replication of *ts* 41 in these mice. Furthermore, preliminary studies have demonstrated *ts* 41 virus in mouse brains 4 to 6 days after i.c. inoculation. Titers of virus were 10^3 to 10^5 $\log_{10}$ PFU per ml of brain (unpublished data).

Mice infected i.c. with wt VSV, *ts* 31, and *ts* 41 were next studied histologically to define the development of pathological changes seen after infection. One day after mice were infected with wt VSV i.c., brains and spinal cords were normal. Two days after infection, however, brains of wt VSV-infected mice demonstrated very rare foci of parenchymal necrosis and more frequent foci of perivascular mononuclear cell infiltration (Fig. 1). These changes were

TABLE 1. *Lethality of wt VSV and ts mutants for 3- to 4-week old Swiss mice after i.c. inoculation*

ts-VSV mutant	LD_{50}[b]-PFU/mouse
III 31	5×10^2
IV 41[a]	$>10^7$
wt	5×10^1

[a] Mice injected with *ts* 41 were clinically well after virus inoculation during 21 days of observation. The *ts* mutants were plaqued at 31 C in BHK-21 cells, whereas wt VSV was plaqued at 37 C.

[b] LD_{50}, Mean lethal dose.

TABLE 2. *Clinical and histological characteristics of i.c. infection with 10 to 100 mean lethal doses of wt VSV, ts 31, or ts 41, in 3- to 4-week-old Swiss mice*

Symptoms	Mice		
	wt VSV	*ts* 31	*ts* 41
Duration of illness (days)	2–3	6–9	None
Wasting	None	Marked	None
Ruffled fur (days present)	Preterminal (h)	3–5	None
Lethargy (days present)	Preterminal (h)	3–5	None
Convulsions	None	Present	None
Hind limb paralysis (days present)	Occasional	3–4	None
Generalized paralysis	Characteristic preterminal	Unusual	None
Histological lesions:			
Parenchymal necrosis	Present, but rare	Present, but rare	None
Perivascular cell infiltrates	Occasional	Occasional	None
Spongiform myelopathy	None	Extensive	None

TABLE 3. *Neutralization of virulence of mice infected with 2 × 10⁶ PFU of ts 31 VSV by anti-VSV antiserum*[a]

No. of mice	Antiserum added	Days after infection at which individual mice died
6		5,5,6,8,8,9
7	NRS (1:50)[b]	6,6,6,6,8,9,9
7	Anti-VSV (1:50)	All survived[c]

[a] Mice were inoculated with 2 × 10⁶ PFU of *ts* 31 after virus and control or anti-VSV antisera were added and allowed to react at 34 C for 60 min.

[b] Normal rabbit serum.

[c] Mice were observed for 21 days and were clinically well throughout.

most evident in the injected hemisphere. The spinal cords of these mice were notable only for loss of cilia in the ependymal cells of the central canal as well as darkening and shrinking of both cytoplasm and nuclei of these cells. No other changes were noted in either the gray or white matter of the spinal cord.

Mice infected with *ts* 31 demonstrated scattered foci of mononuclear inflammatory cells around small blood vessels in the brain by 4 days after infection. In addition, mononuclear cell infiltrates were also seen around the lateral ventricles, especially in areas where the ependymal lining appeared dark and shrunken in appearance. Most striking, however, were the spinal cord lesions seen in *ts* 31-infected mice. By 4 days after infection, the ependymal cells of the central canal had lost their cilia, appeared dark and shrunken, and often were frankly necrotic. Most unexpected, however, were the striking spongiform changes evident in the gray matter of the spinal cords by 4 days after infection. At this time, the spongiform changes were localized to the anterior horns. By 5 days after infection the spongiform changes were more extensive, being present throughout gray matter of the spinal cord (Fig. 2). These spongiform changes were due to dilatation principally of perivascular (Fig. 3 and 4) and perineuronal processes (Fig. 4), although other areas of the neuropil were also affected. Swollen processes could reach considerable dimensions, often exceeding the size of large neurons. Little inflammation accompanied the spongiform changes initially (4 days), but by 5 days after infection mononuclear cell infiltration was evident. The white matter of the spinal cord was unremarkable and no intracytoplasmic or intranuclear inclusion bodies were observed. Preliminary ultrastructural studies suggested that these enlarged, swollen processes were mainly of astrocytic and dendritic origin.

Light and electron microscope examinations were also performed for mice infected i.c. with *ts* 41 VSV. Brains and spinal cords were examined 4 and 8 days after infection and appeared entirely normal. Thus, after i.c. inoculation of *ts* 41 no pathological lesions were evident.

DISCUSSION

The i.c. injection of wt VSV produces a rapidly fatal encephalitis with death of mice in 2 to 3 days. Histologically, such mice exhibit minimal changes of encephalitis (Fig. 1). These changes include rare areas of parenchymal necrosis and more frequent foci of mononuclear cell infiltration surrounding small vessels in the brain. In contrast to the highly virulent, rapidly fatal CNS infection seen after i.c. inoculation of wt VSV, infection with *ts* 31 VSV produced a slowly progressive CNS infection characterized by previously unreported spongiform myelopathy (Table 2 and Fig. 2–4).

These CNS clinical and histopathological changes appear unique to the *ts* 31 mutant of VSV. For example, UV inactivation of *ts* 31 virus results in no clinical disease and, furthermore, i.c. inoculation of *ts* 41 VSV results in

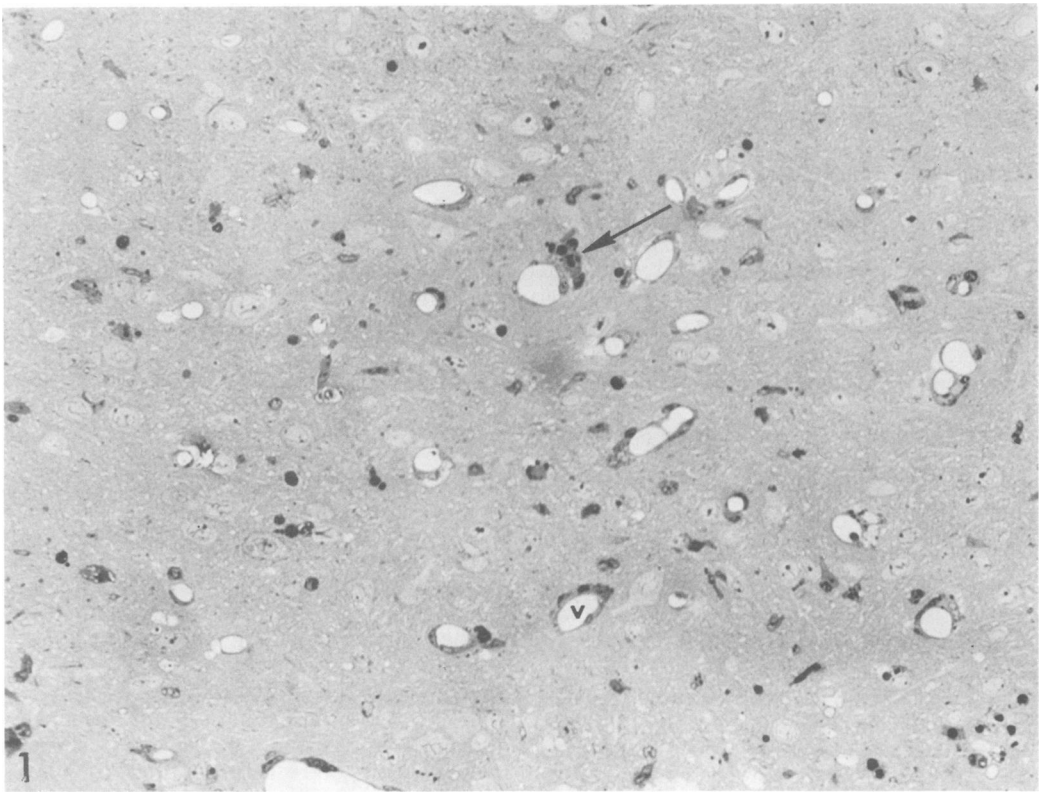

FIG. 1. *Cortical section from a mouse 2 days after inoculation of wt VSV. Several vascular channels are present showing active endothelial cells and pericytes. The arrow points to a group of mononuclear inflammatory cells in relation to one of the vessels. Similar cells are also scattered in the neuropil. V is in the lumen of one of the vessels. Epon-embedded section, 1 μm thick. ×310.*

neither clinical illness nor histopathological lesions (Table 2). Thus, the slowly progressive CNS disease and spongiform myelopathy seem associated with the host-parasite relationship established with this *ts* mutant of VSV.

Several reservations, however, must be raised in this regard. First, it is not clear from our studies that wt VSV-infected mice would not develop the histopathological changes observed after infection with *ts* 31 if the mice survived longer than 2 to 3 days. Thus, although we have never observed spongiform myelopathy in weanling Swiss mice infected i.c. with wt VSV, we cannot be sure that the spongiform changes would not occur with wt VSV infection. Although these spongiform changes may occur with wt VSV infection, previous studies on the histopathological changes seen in the CNS with wt VSV infection do not report such findings (7). Second, preliminary work with another *ts* mutant of VSV (*ts* 22, complementation group II) suggests that this mutant may also be associated with slowly progressive CNS disease and spongiform myelopathy (unpublished data). Thus, it is not entirely clear at this time whether the slowly progressive CNS disease and spongiform lesions are unique to the *ts* 31 mutant of VSV or whether other *ts* mutants of VSV also have this capability. What is clear, however, is that the *ts* 31 mutant of VSV, and perhaps other *ts* mutants, possesses the capability of radically altering CNS disease usually associated with VSV infection. In addition, the *ts* mutants appear capable of producing histopathological lesions which have some similarities to the so-called slow viral diseases such as Creutzfeldt-Jakob disease, Kuru, and scrapie (1, 5). These studies therefore, support the contention that *ts* mutants may have a role in vivo in viral diseases of altered pathogenicity. In addition, recent studies by Haspel et al. (3) with *ts* mutants of measles virus in a hamster model support the contention that certain *ts* mutants may possess the capacity to alter rapidly fatal wt virus infection into a more slowly progressive infection. Furthermore, *ts* mutants of VSV may serve as useful probes to study the biochemical defects

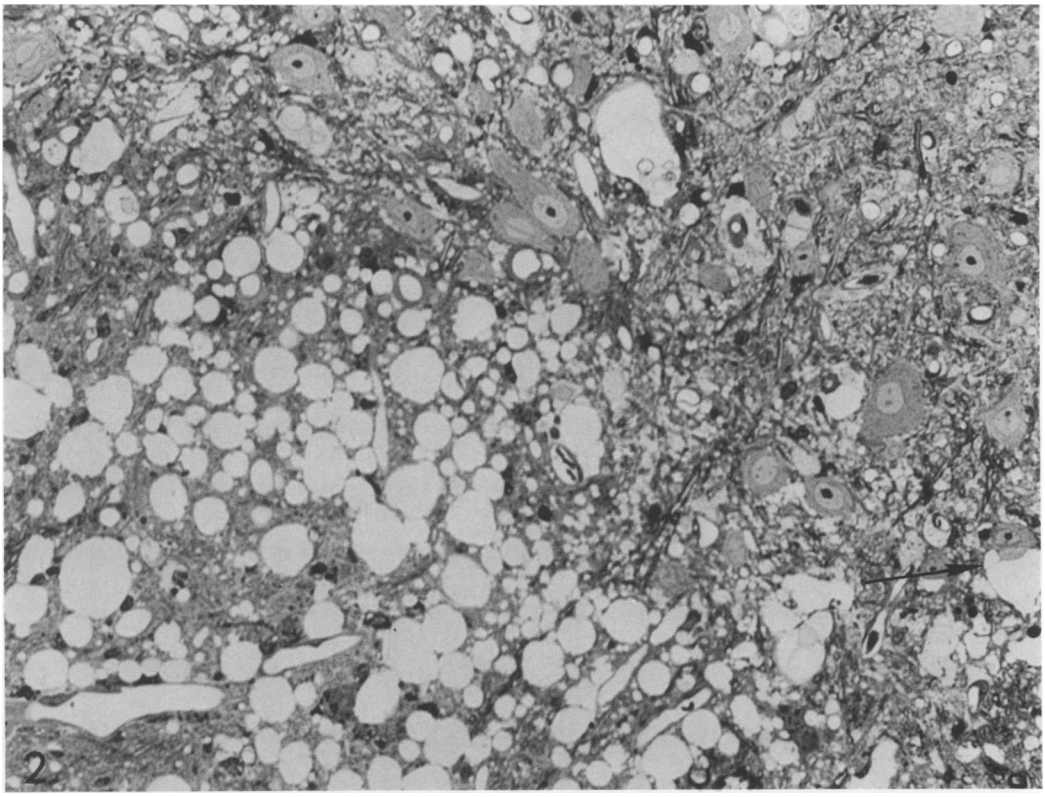

FIG. 2. *The anterior horn of the spinal cord shown in this section was taken from a mouse 5 days after i.c. inoculation of ts 31. Note the presence of numerous vacuoles which give a spongiform appearance to this field. Where vacuoles are most numerous, neurons are generally not identifiable by light microscopy. The arrow points to a large vacuole in contact with a still well-preserved neuron. Epon-embedded section, 1 μm thick. ×310.*

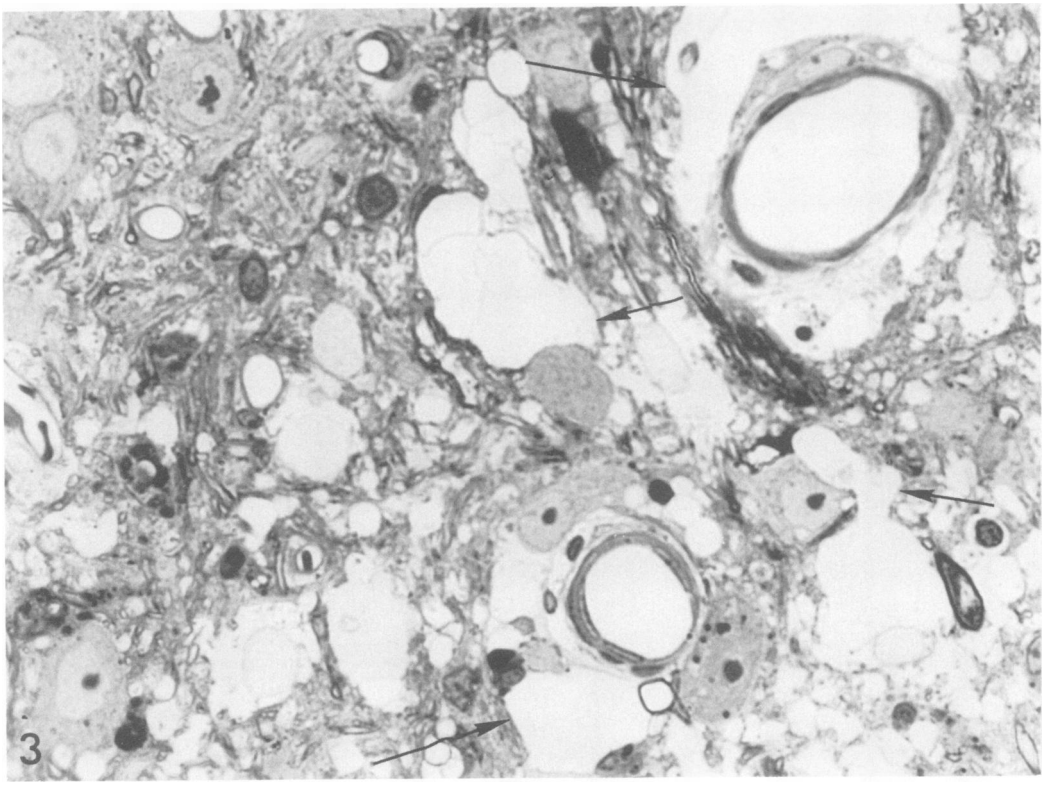

FIG. 3. *Higher-power view of an anterior horn moderately involved by spongiform changes, showing large perivascular (long arrows) and perineuronal (short arrows) vacuoles. Septa inside some of the larger vacuoles indicate that multiple contiguous processes may participate in their formation. Section of spinal cord taken 5 days after inoculation i.c. of ts 31. Epon-embedded section, 1 μm thick. ×770.*

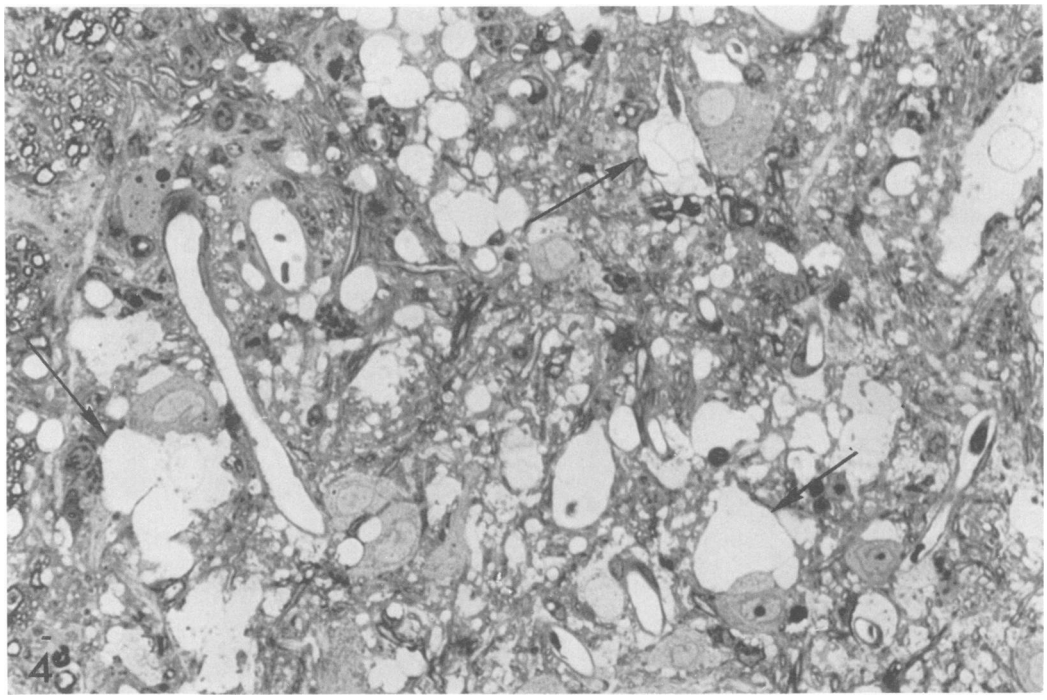

FIG. 4. *Section from anterior horn taken 5 days after ts 31 inoculation, showing numerous vacuoles in the neuropil (top center) as well as large perineuronal vacuoles (arrows). Epon-embedded section, 1 μm thick. ×480.*

in virus replication and altered host-virus relationships.

Finally, these studies suggest that use of *ts* mutants as vaccines may be associated with significant hazards. Use of such *ts* mutants has already been reported for influenza (6) and respiratory syncytial virus (2). Use of such mutants has a number of theoretical advantages, including their ability to replicate to high titer at permissive temperatures (31 to 33 C) but only to a limited extent at nonpermissive temperatures (37 to 39 C). In addition, replication of these mutants at permissive temperatures to high titer assures synthesis of viral proteins in sufficient concentration to allow substantial host immune reponses to occur. Such host responses are essential to active sensitization and the desired result of long-lasting and durable immune responses. The major problem associated with use of these *ts* mutants is that they have not been genetically characterized. Thus, serious questions must be raised with regard to their genetic stability and, hence, safety. Our studies also suggest that for at least VSV, certain *ts* mutants may be associated with a significantly altered clinical illness from that seen with ordinary wt infection. These observations raise the question as to whether *ts* mutants might not also have an important role to play in vivo in slowly progressive or unusual viral syndromes.

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