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STATUS SPONGIOSUS RESULTING FROM INTRACEREBRAL INFECTION OF MICE WITH TEMPERATURE-SENSITIVE MUTANTS OF VESICULAR STOMATITIS VIRUS

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Summary.—Mice infected intracerebrally (i.c.) with wild-type (wt) VSV or temperature-sensitive (*ts*) mutants, *ts* 11, *ts* 22, *ts* 31 and *ts* 41, were studied for the development of histopathological lesions in the central nervous system (CNS). Mice infected i.c. with wt VSV exhibited histopathological lesions consisting principally of occasional foci of perivascular mononuclear cell infiltration and rare foci of necrosis. All wt VSV infected mice died within 2 days of i.c. inoculation. In contrast, mice infected i.c. with *ts* 22 and *ts* 31 developed spongiform lesions limited to the grey matter of the spinal cord beginning 4 days after inoculation. The spongiform lesions rapidly spread to involve the entire grey matter of the spinal cord by 5–7 days after infection. Vacuolar changes were restricted principally to neuronal processes and astrocytes. *Ts* 22 and *ts* 31 infected mice developed neurological illness beginning 4 days after infection and the majority of mice died by 7 days after infection. Mice infected with *ts* 11 and *ts* 41, on the other hand, remained clinically well and were devoid of neuro-pathological lesions at 4 and 8 days after infection.

WITHIN the past decade, considerable interest has been directed at studying temperature-sensitive (*ts*) mutants of viruses (Burge and Pfefferkorn, 1966, 1968; Simpson and Hirst, 1968; Fenner, 1969; Gharpure, Wright and Chanock, 1969; Tan, Sambrook and Bellett, 1969; MacKenzie, 1970; Pringle and Duncan, 1971). *Ts* mutants of a variety of animal viruses have been used to probe viral replication, biochemistry and genetics. Recently, however, several laboratories have reported on *in vivo* studies utilizing *ts* mutants and parental wild-type (wt) virus to probe the effects of *ts* mutants on viral pathogenesis (Fields, 1972; Shaver and Van Bekkum, 1972; Ter Meulen *et al.*, 1972). Some of the *ts* mutants have shown the capacity to markedly alter the clinical pattern of infection and, in fact, to produce entirely different histopathological lesions than associated with parental wt infection.

We have recently become interested in studying the *in vivo* capacity of *ts* mutants of vesicular stomatitis virus (VSV) to produce central nervous system (CNS) infection. As reported by others (Miyoshi, Harter and Hsu, 1971) and confirmed by us (Rabinowitz, Dal Canto and Johnson, 1976), wt VSV CNS infection is characterized by a rapidly fatal encephalitic-type illness associated with a paucity of histological lesions in the CNS of affected mice. In contrast, we have already reported on the altered clinical pattern of infection produced by certain *ts* mutants of VSV. Following intracerebral (i.c.) inoculation of *ts* 31 VSV, for example, weanling Swiss mice develop hind limb paralysis beginning 4–5 days after infection which lasts 3–4 days

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before the mice die. Wt-VSV infected mice, on the other hand, die without obvious neurological signs, except preterminally, in 2–3 days.

We now wish to report on the development of histopathological lesions in brain and spinal cord of weanling Swiss mice infected with wt VSV as well as 4 *ts* mutants of VSV, i.e. *ts* 11, *ts* 22, *ts* 31 and *ts* 41. The 4 *ts* mutants represent members of VSV complementation groups I–IV as defined by Pringle (Pringle, 1970*a*).

Our studies indicate that in contrast to wt VSV, CNS infection with *ts* 31 and *ts* 22 but not *ts* 11 or *ts* 41 is associated with previously unreported extensive spongiform lesions in the grey matter of the spinal cord of mice. Furthermore, these lesions appear 4 days after intracerebral (i.c.) inoculation and spread rapidly throughout the grey matter of the spinal cord until by 5–7 days after infection almost all of the grey matter is involved. The extensive spongiform myelopathy produced by certain *ts* mutants of VSV may provide a useful model for probing altered host–virus relationships related to biochemical defects in virus replication.

MATERIALS AND METHODS

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

Viruses.—Indiana strain (wt) VSV was plaque-purified and doubly-cloned as previously described (Rabinowitz *et al.*, 1976). *Ts* mutants 11, 22, 31 and 41 were generously provided by Dr M. E. Reichmann (University of Illinois, Urbana, Illinois). These *ts* mutants have previously been extensively described (Pringle, 1970*b*; Reichmann, Pringle and Follett, 1971). Methods for growing and titering each of *ts* mutants as well as wt VSV have previously been described (Rabinowitz *et al.*, 1976).

Wt VSV titered 2×10^9 PFU/ml in BHK-21 cells when plaqued at 37°. *Ts* mutants, on the other hand, were plaqued at 31° in BHK-21 cells and titered: (1) *ts* 11—$2{\cdot}25 \times 10^9$ PFU/ml; (2) *ts* 22—$3{\cdot}35 \times 10^7$ PFU/ml; (3) *ts* 31—$4{\cdot}7 \times 10^7$ PFU/ml; (4) *ts* 41—$5{\cdot}55 \times 10^8$ PFU/ml.

Infection.—Mice were lightly anaesthetized and injected in the right parietal region with the following amounts of virus; (1) wt VSV—1×10^3 PFU; (2) *ts* 11—$7{\cdot}5 \times 10^5$ PFU; (3) *ts* 22—1×10^4 PFU; (4) *ts* 31—1×10^4 PFU; (5) *ts* 41—$1{\cdot}7 \times 10^5$ PFU. In the case of wt VSV, *ts* 22 and *ts* 31, these doses represented 10–100 50%-lethal doses (LD_{50}) when given i.c. *Ts* 11 and *ts* 41 were avirulent even when inoculated i.c. in doses 10^7 PFU.

Histopathological studies.—Mice inoculated with wt VSV were killed each day. By 2 days these mice were already moribund. Mice inoculated with *ts* 22 and *ts* 31 were killed 2, 3, 4, 5 and 7 days after infection. Two mice were sacrificed for each mutant per day of study. Beginning 4 days after infection *ts* 22 and *ts* 31 infected mice exhibited hind-limb paralysis and the majority died by 7 days. *Ts* 11 and *ts* 41 infected mice were sacrificed 4 and 8 days after infection. Again 2 mice per mutant were killed for each day of study.

The mice were anaesthetized by i.p. injection of 0·02 ml Diabutal (50 mg/ml) and then perfused *via* the left ventricle with chilled (4°) 3% glutaraldehyde in phosphate buffer (PB), (pH 7·4). Brain and spinal cord were dissected out and small sections were postfixed in 1% osmic acid in PB, dehydrated through a graded series of concentrations of ethyl alcohol, cleared in propylene oxide and embedded in Epon. One-micron sections were prepared, stained with toluidine blue, and observed by light microscopy.

RESULTS

Wt VSV

Histopathological lesions were first evident 2 days after i.c. inoculation of wt VSV. There were foci of perivascular and parenchymal mononuclear cell infiltration in the brain, particularly in regions close to the ventricles (Fig. 1). Ependymal cells by 2 days after infection were shrunken, had dark cytoplasm and appeared to be detached from the ventricular wall. Rare areas of focal parenchymal necrosis were also observed. Most of the necrotic lesions appeared in the right hemisphere, the side of inoculation.

In the spinal cord, the only changes observed were also first apparent two days after infection. Lesions in the spinal cord were limited to the central canal. Ependymal cells appeared shrunken and, in general, demonstrated findings

analogous to those observed in ependymal cells of the brains of wt-infected mice (Fig. 2). When ependymal necrosis had developed, the central canal appeared either partially or completely disrupted. Of note was the fact that no changes were evident in either the grey or white matter of the spinal cord.

Ts 22 *and ts* 21

In contrast to wt VSV infection, the first pathologic changes after i.c. inocu-

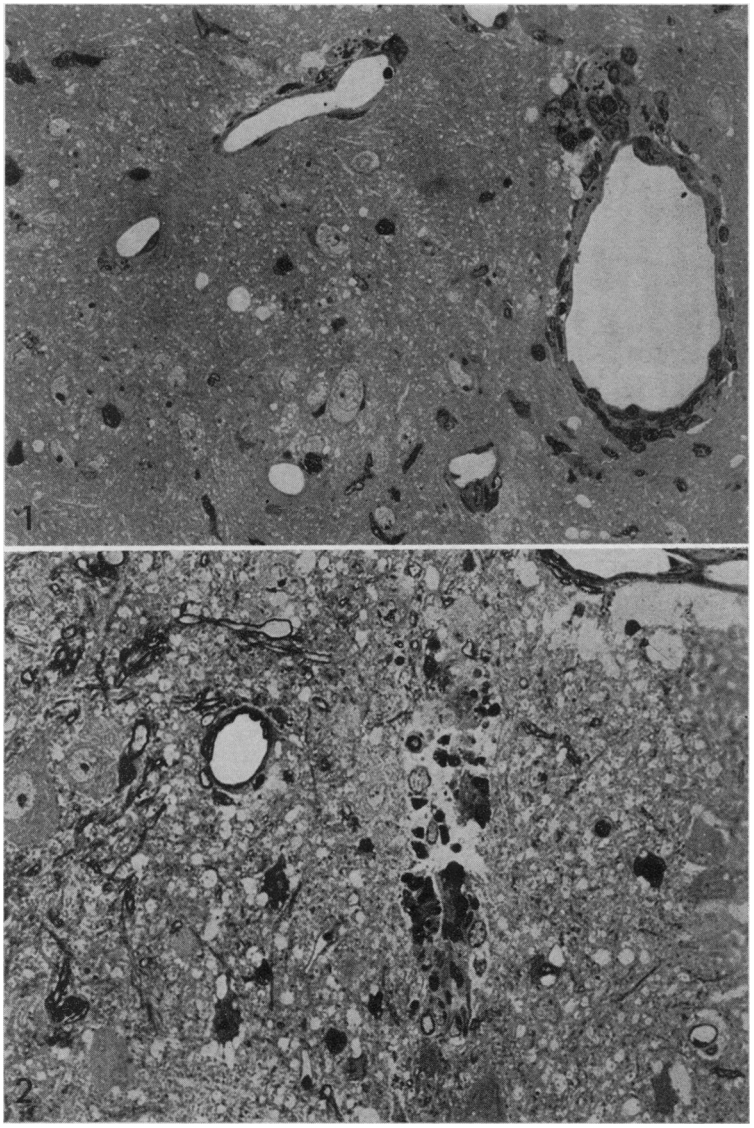

FIG. 1.—Section from deep cortex 2 days after inoculation of wt VSV. There is perivascular infiltration by mononuclear cells, most of which appear to be of the microglial series. Similar cells are also scattered in the neuropil. Epon embedded 1 μm thick section, stained with toluidine blue. $\times 345$.

FIG. 2.—Section from spinal cord 2 days after inoculation of wt VSV. The central canal is disrupted due to degeneration and detachment of most ependymal cells. No other pathologic changes are otherwise observed. Epon embedded 1 μm thick section, stained with toluidine blue. $\times 400$.

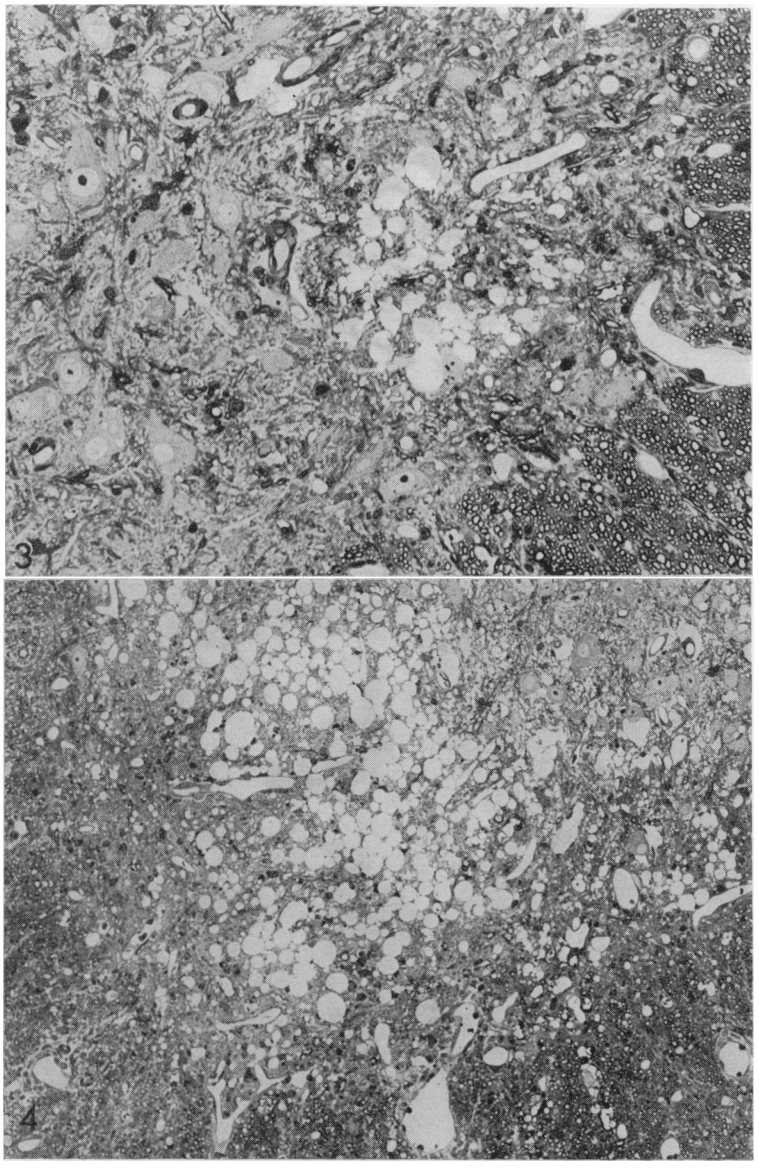

FIG. 3.—Section from spinal cord 4 days after inoculation of *ts* 31. The central portion of the anterior horn shows the presence of numerous well defined vacuoles of various dimensions. No inflamation is present at this early stage. No other pathologic changes in either grey or white matter are seen. Epon embedded 1 μm thick section, stained with toluidine blue. $\times 240$.

FIG. 4.—Section from spinal cord 5 days after inoculation of *ts* 31. Spongiform changes are now involving the entire grey matter and a few mononuclear cells are also present among the vacuoles. Epon embedded 1 μm thick section stained with toluidine blue. $\times 175$.

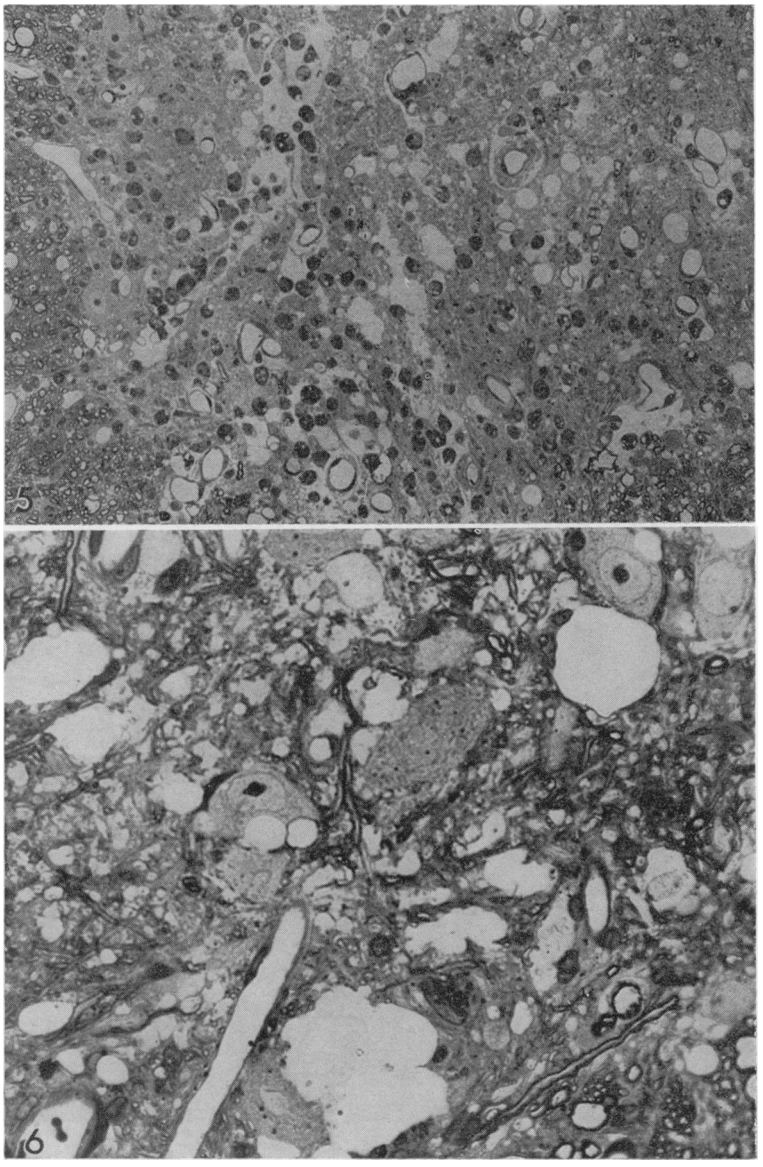

FIG. 5.—Section from spinal cord 7 days after inoculation of *ts* 31. A few well defined vacuoles are still present, but the overall appearance is now that of neuropil disorganization and mononuclear cell infiltration. Several macrophages are also seen. Epon embedded 1 μm thick section stained with toluidine blue. $\times$ 185.

FIG. 6.—Higher power view of spinal cord 5 days after inoculation of *ts* 22. Vacuoles are seen abutting on the neuronal plasma membranes. The neuron at the bottom of the picture is surrounded by a very large vacuole in which remains of membranes are visible suggesting coalescence of several swollen profiles. Epon embedded 1 μm thick section, stained with toluidine blue. $\times$ 640.

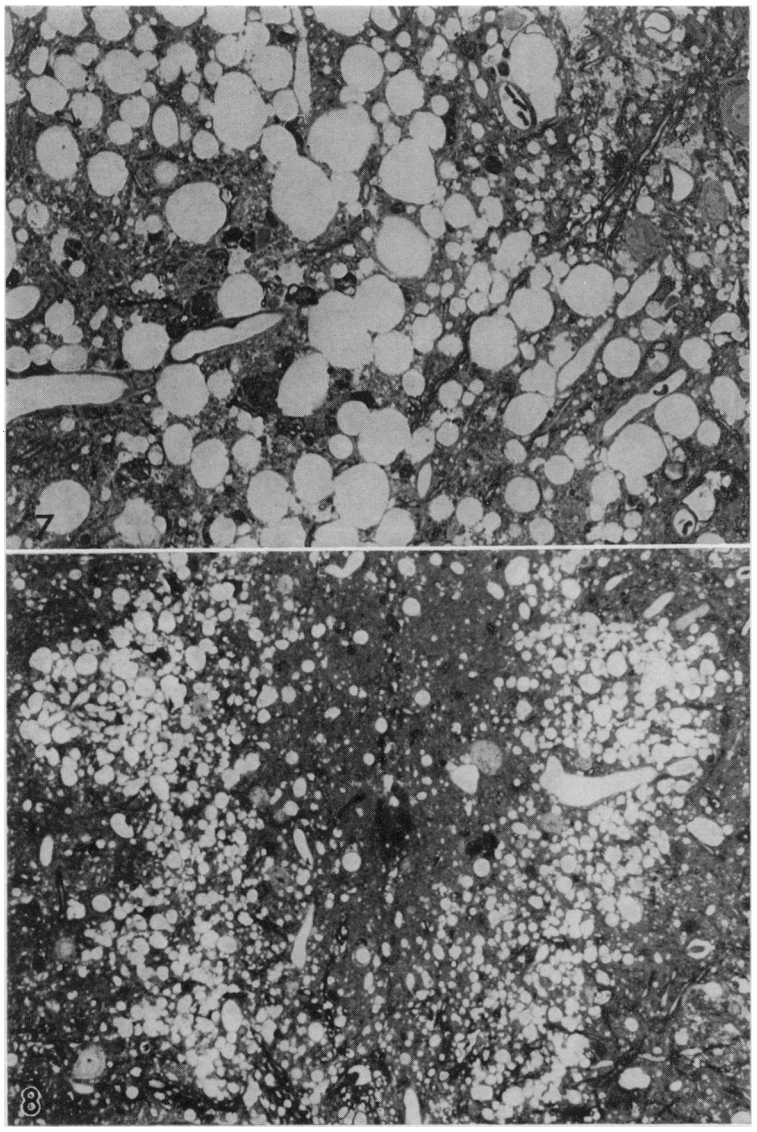

FIG. 7.—Section from spinal cord 5 days after inoculation of *ts* 31. Several capillaries and venules are seen in this field and vacuoles often abut on their walls. Epon embedded 1 μm thick section, stained with toluidine blue. $\times 400$.

FIG. 8.—Section from spinal cord 4 days after inoculation of *ts* 22. The dark cells at the centre are shrunken ependymal cells, which are surrounded by concentric rows of vacuoles. This peculiar pattern was often seen at 4 days after infection with both *ts* 22 and *ts* 31 mutants. Concomitant beginning vacuolization in the anterior horns could sometimes be observed. Epon embedded 1 μm thick section stained with toluidine blue. $\times 160$.

lation of *ts* 22 and *ts* 31 appeared 4 days after inoculation. The lesions in the brain were essentially the same as those described for wt VSV infection. The striking new findings, however, were observed in the spinal cord. Beginning 4 days after inoculation, spongiform lesions appeared in the spinal cord (Fig. 3). At first these spongiform lesions appeared in the anterior horn, but with time spread to involve the entire grey matter (Fig. 4). When the spongiform lesions were first evident, no inflammatory cells were noted (Fig. 3). By the time the status spongiosus had spread to involve the entire grey matter of the spinal cord, inflammatory cells were present (Fig. 4). Seven days after inoculation of *ts* 22 and *ts* 31, the extensive spongiform degeneration had largely been replaced by diffuse disorganization of the architecture of the grey matter (Fig. 5).

The spongiform lesions consisted of vacuoles varying in size from those just visible at the light microscopic level to those 2–3 times the size of large anterior horn neurons. Many such vacuoles were seen around neurons (Fig. 6). In some cases these vacuoles abutted on and deformed the cytoplasmic membrane of neurons, while other vacuoles were seen around vascular channels of various sizes (Fig. 7). Vacuoles were also seen in the neuropil at sites removed from neurons and vessels. Often, large vacuoles demonstrated internal septa which represented areas where the membranes of multiple swollen processes appeared to coalesce. Presumably, with rupture of these membranes, fusion of processes could take place.

A peculiar pattern was often observed in the area around the ependyma 4 days after inoculation with both *ts* 31 and *ts* 22 mutants. It consisted of concentrically placed vacuolar changes around the central canal which was smaller than normal and lined by abnormally dense ependymal cells (Fig. 8). By 5–7 days after infection, the central canal was sometimes completely obliterated but ependymal necrosis was very rarely seen.

The white matter of spinal cord remained normal throughout the 7 days of study. At no time was vacuolization evident in the white matter. Thus the lesions appeared to have strict specificity for the grey matter of the spinal cord.

Ts 11 *and ts* 41

Examination of the brain and spinal cord of mice infected i.c. with either *ts* 11 or *ts* 41 failed to demonstrate any neuropathological lesions. Brains and spinal cords were examined histologically 4 and 8 days after i.c. inoculation of the mutants and were devoid of any lesions. Thus a striking dichotomy appeared to exist between the capacity of *ts* 22 and *ts* 31 as compared with *ts* 11 and *ts* 41 to produce spongiform myelopathy.

DISCUSSION

Ts 22 and *ts* 31 inoculation i.c. in mice induced a neurological illness characterized by slowly progressive CNS dysfunction and striking spongiform myelopathy. Wild-type VSV infection, on the other hand, produced fulminating CNS infection with death of mice in 2–3 days. Pathologically, changes in the ependyma of the central canal were the earliest histological finding with both wt and *ts* VSV infection. These changes suggest that virus inoculated i.c. spreads rapidly to the spinal cord. Although changes in the ependymal lining of the spinal cord were evident with wt VSV, *ts* 22 and *ts* 31, only infection with the *ts* mutants produced striking spongiform myelopathy. It is difficult at this time to be sure, however, that the spongiform myelopathy is unique to *ts* VSV infection, or also possible with wt VSV. It may be possible that spongiform myelopathy is not observed with wt VSV infection because the host dies before histopathological lesions have time to develop.

The vacuoles seen following *ts* 22 and *ts* 31 CNS infection appear to be mainly perineuronal and perivascular in location,

which suggests that the vacuoles are mainly dendritic and astrocytic in origin. Preliminary ultrastructural study of these vacuoles would support this possibility (unpublished data). Additionally, vacuoles are occasionally located within myelin sheaths. Intraneuronal vacuoles, on the other hand, appear to be present only occasionally, and when present are of small size.

While *ts* 22 and *ts* 31 are associated with striking spongiform myelopathy, CNS infection with *ts* 11 and *ts* 41 are not. In fact, inoculation of these mutants is neither associated with clinical illness nor histopathological lesions. At first, we considered the reason for the avirulence observed after i.c. inoculation of *ts* 11 and *ts* 41 to result from the inability of these mutants to replicate at non-permissive temperatures *in vivo*. But, *ts* 11 and *ts* 41 VSV can be recovered from brains of infected mice 4–6 days after i.c. inoculation, in titres varying from $1{\cdot}0 \times 10^3$–$1{\cdot}0 \times 10^6$ PFU/ml brain (unpublished data). Thus it appears as if both *ts* 11 and *ts* 41 have the ability to replicate to some extent in the CNS of mice. Further work is under way to define the mechanism by which certain *ts* mutants of VSV produce spongiform lesions.

The histopathological findings observed with certain *ts* mutants of VSV invite comparison with other natural and experimental conditions associated with status spongiosus. Type C virus in mice is associated with motor neuron degeneration and some degree of status spongiosus in both grey and white matter (Andrews and Gardner, 1974). In this infection, vacuoles are found in neurons as well as non-neuronal cells. Most of the vacuoles, however, appear in the cytoplasm of unidentified cells scattered through the CNS (Andrews and Gardner, 1974). In addition, Zlotnik and Grant (1975) have described vacuolization induced in both grey and white matter of the CNS following inoculation of Langat virus and of a hamster-adapted measles virus in hamsters. In both viral infections, however, the degree of vacuolization is of a much more limited extent than we describe for *ts* VSV infection. Moreover, CNS infection with Langat virus or measles virus does not produce vacuoles with a striking predilection for the grey matter or for the spinal cord.

Recently Takayama and Nakano (1975) described spongy changes occurring diffusely in the hippocampus, thalamus and occasionally in cortex of mice inoculated i.c. with a *ts* mutant of western equine encephalitis virus (WEE). In these lesions nerve cells as well as glial cells were decreased in number and in some areas nerve cells disappeared completely. In addition, considerable inflammation accompanied the spongy changes. Moreover, both parental wt WEE as well as a *ts* WEE mutant produced these changes. Finally, lesions involved both the grey matter as well as the white matter.

Status spongiosus has also been observed after administration of various chemical agents. Cuprizone (Suzuki and Kikkawa, 1969), isonicotinic acid hydrazide (Lampert and Schochet, 1968), hexachlorophene (Kimbrough and Gaines, 1971) and triethyltin sulphate (Aleu, Katzman and Terry, 1963) are associated with intramyelinic vacuoles caused by splitting of the intraperiod line. The spongy changes in these conditions are therefore mainly localized in the white matter of the CNS. Canavan's spongy degeneration, a natural human disease, is also characterized by status spongiosus of the white matter in a pattern similar to that seen with the above mentioned chemical agents.

Finally, status spongiosus has been associated with a group of diseases of man and experimental animals known as subacute viral spongiform encephalopathies (Lampert, Gajdusek and Gibbs, 1972). Diseases in this category include Creutzfeldt–Jakob disease and Kuru in man and scrapie and transmissible mink encephalopathy in animals. These diseases are considered of viral origin

because they can be transmitted to animals with bacteria-free materials obtained from infected animals. However, the physical-chemical nature of these infectious agents is unknown at the present and most likely their properties are distinctive from those of known viruses.

Subacute viral spongiform encephalopathies have extensive spongiform degeneration principally involving neuronal perikaria and processes although astrocytes may also be involved (Gonatas, Terry and Weiss, 1965; Lampert *et al.*, 1969). In some respects the lesions we have described with *ts* VSV infection resemble what is described with the subacute viral spongiform encephalopathies. The principal areas of similarity include: (1) involvement of neuronal processes and astrocytes; (2) lesions confined to the grey matter; (3) association of lesions with virus or virus-like agent. There are, however, two major differences between VSV-induced spongiform changes and the subacute spongiform encephalopathies. These differences are: (1) absence of astrocytosis in VSV infection; (2) presence of inflammation in VSV lesions. Nevertheless, the experimental disease we have described represents a new model for status spongiosus. This model appears to offer new insights into the nature of host–virus relationships as they affect the pathogenesis of virus infection.

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