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Pathogenicity of an Attenuated, Temperature-Sensitive Mutant of Western Equine Encephalitis Virus Induced by a Chemical Mutagen

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To know the pathogenicity of the chemically induced, temperature-sensitive (ts) mutant of western equine encephalitis virus, designated tsNG39, the lethality for mice injected with tsNG39, virus yield, interferon production, and histological changes in the brains of these mice were examined in parallel with those of mice inoculated with the parent strain (PS). All of the mice injected intracranially with PS died within 3.5 days after injection irrespective of the inoculum size of virus, whereas the lethality of the mice inoculated with tsNG39 varied from 94.3 to 65.5% among groups of mice and this variation seemed to be correlated with the inoculum size of virus rather than with the maximum virus titer in the brain. By histological examination, two types of changes in the brain were distinguished, inflammatory and degenerative ones. Inflammatory changes were more prominent in the brains injected with tsNG39 than in those receiving PS. Degenerative changes were dominant in the brains injected with PS, but they were slight in the earlier phase of infection by tsNG39 and became prominent only later. The degree of degenerative change was well correlated with both the virus titer in the mouse brain and the death pattern of mice injected with PS or tsNG39. Since degenerative changes are thought to be caused by the direct effect of injected virus, these results indicated that the factor responsible for the low virulence of tsNG39 was the slow viral growth in the brain.

The isolation of a chemically induced, temperature-sensitive (ts) mutant of Western equine encephalitis virus has been reported (14). This mutant, designated tsNG39, could produce no infectious particle in cells incubated at the nonpermissive temperature of 42 C, although it replicated at 37 C (permissive temperature) as did the parent strain (PS). The PS grew normally at both 37 and 42 C. When tsNG39 was inoculated into the mouse brain, it showed a lower viral yield than that of the PS. The mice inoculated with tsNG39 survived longer than those injected with PS, but a bizarre death pattern was observed in the mice that received a low dose of tsNG39 (14).

To elucidate the causes of this irregular death pattern and to explore further the pathogenicity of this ts mutant, the cumulative death curve of the mice receiving tsNG39, the growth curve of the mutant in mouse brains, and interferon (IF) production in the brain were examined and compared with those of mice injected with the PS. Histological changes of the mouse brain were compared among groups of mice infected with either PS or tsNG39, because it was of interest to know if the grade of histological change had a correlation to the viral yield or to the lethality of the host animal.

MATERIALS AND METHODS

Cell cultures. Primary chicken embryo cell cultures were prepared by trypsinization of 10-day-old eviscerated embryos. Chicken embryo cell cultures were grown in Eagle minimum essential medium (MEM; Nissui Seiyaku Co., Tokyo, Japan) containing 60 μg of kanamycin per ml supplemented with 5% calf serum (CS). In some experiments Japanese quail embryo cells as well as chicken embryo cells were used for the plaque assay of viruses, since quail embryo cells were found to be comparable to chicken embryo cells in regard to virus yield and sensitivity of the plaque assay (16). L cells were cultured in the same medium with 10% CS. For IF assay L cells were planted in 3-cm glass petri dishes and grown in an atmosphere of 2 to 3% CO_2.

Viruses. The McMillan strain of Western equine encephalitis virus and its ts mutant, tsNG39, were used in this experiment. The latter had been isolated by treatment of the wild-type strain with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, 200 μg/ml (14). The Indiana strain of vesicular stomatitis virus prepared in chicken embryo cells was used for mouse IF

assays. A reference mouse IF was prepared by infecting L cells with the Miyadera strain of Newcastle disease virus.

Mice. Outbred 4- to 5-week-old male Swiss mice were obtained locally (Shiihashi Co., Tokyo, Japan).

Inoculation of virus and preparation of brain tissue emulsion. Viruses were diluted appropriately in MEM with 5% CS, and 0.02 ml of each dilution was inoculated into the right frontal lobe of mice. As a control the same amount of MEM added with 5% CS was similarly injected into some mice. For cumulative mortality studies mice were observed every 12 h for neurological signs and death. At indicated intervals groups of two mice were sacrificed; brains were removed, pooled, ground with sterile sand, and suspended in 7 ml of MEM with 10% CS to obtain 10% (wt/vol) emulsion of mouse brain (brain weight was estimated to be 0.35 g). All brain emulsions were clarified by centrifugation at 2,000 rpm for 10 min, and the supernatant fluids were saved. Each supernatant was divided into two parts; one was used for virus assay and the other was provided for IF assay, and the former was stored at −20 C until titration was performed.

Plaque assay. Plaque assay of virus samples were done as described previously (15). Virus titers in plaque-forming units (PFU) were determined after a 2-day incubation at 37 or 42 C. The incubation at 42 C was performed in a constantly stirred water bath controlled at 42 ± 0.1 C.

Assay of mouse brain IF. To inactivate the virus in brain emulsion, its pH was brought to about 2 by adding N HCl and left at 4 C for 4 days, and then it was neutralized with N NaOH and stored at 4 C until assayed. For the IF assay of brain emulsion a serial fourfold dilution in MEM with 5% CS was made, and 0.9-ml aliquots of each dilution were inoculated onto triplicate monolayers of L cells. Monolayers were incubated at 37 C overnight in an atmosphere of 2 to 3% CO_2 and then challenged with 50 to 100 PFU of vesicular stomatitis virus. After a virus adsorption at 37 C for 60 min, cells were overlaid with the nutrient agar (15) and incubated at 37 C for 2 days. The IF titer was expressed as the 50% plaque-depressing dose obtained by the graphic method according to Lindenmann and Gifford (11).

Statistics. Probit analysis was used to calculate 50% death time of mice and its standard deviation (18).

Histological procedure. Mice were injected with PS or tsNG39, as described above, and sacrificed to remove their brains at an appropriate time postinfection. Brains were dissected into five pieces and embedded in paraffin wax. Sections were stained by the hematoxylin-eosin and Kluver-Barrera methods. To illustrate graphically the histological results, separate estimates were made for the degenerative and inflammatory changes. Four grades were adopted to show the degree of each of the pathological processes. The description of these changes was concerned with following criteria. (i) Degenerative changes, mainly spongy: grade I, slight vacuolic change of ground substance; grade II, localized focal spongy change with slight degenerating nerve cells; grade III, multiple focal spongy change with moderate degenerating nerve cells; grade IV, widespread spongy change with severe degenerating nerve cells. (ii) Inflammatory changes, mainly perivascular cuffing: grade I, slight perivascular cuffing composed of one layer of infiltrating cells; grade II, widespread slight perivascular cuffing; grade III, widespread moderate perivascular cuffing, composed of several layers of infiltrating cells; grade IV, widespread severe perivascular cuffing, composed of many layers of infiltrating cells.

RESULTS

Clinical manifestations of mice after intracranial inoculation. The mice intracranially injected with PS developed loss of appetite and movement, general tonic convulsions, and finally death. The mice inoculated with tsNG39, however, showed different reactions, even though they were given the same PFU of virus. Some of them became very sensitive to slight stimuli, some assumed a hunched posture and had ruffled fur, and others appeared almost healthy. Generally tonic convulsions were observed in the sick mice just before their death. No clinical sign was observed in the mice inoculated with the ultraviolet-inactivated PS or tsNG39, or in the mice injected with the same volume of diluent (MEM added with 5% CS).

Comparative death pattern of PS and tsNG39 by intracranial inoculation. To discover a correlation between the death of mice and the inoculum size of virus, and to clarify the cause of the irregular death pattern of the mice injected with tsNG39 that was observed in a previous experiment (14), groups of 15 to 30 mice were intracranially inoculated with PS using 10^7, 10^5, or 10^3 PFU/0.02 ml (each group of mice was designated P-10^7, P-10^5, and P-10^3, respectively) or injected with tsNG39 using 10^7, 10^5, or 10^3 PFU (named ts-10^7, ts-10^5, and ts-10^3, respectively). Each cumulative death pattern was determined based on the results of three repeated experiments (Fig. 1).

The cumulative death patterns of the P-10^7, P-10^5, and P-10^3 groups were about the same and they seemed to fit the cumulative curve of normal distribution, because each of the cumulative percent death curves fit a straight line on the probit scale paper. Fifty percent death times of these three groups, represented by five probits, were 2.3, 2.4, and 2.4 days, respectively, and their standard deviations were about 0.6 day. The mice inoculated with tsNG39, on the other hand, did not die before day 3 after injection, and they showed quite a different death pattern from that of the mice

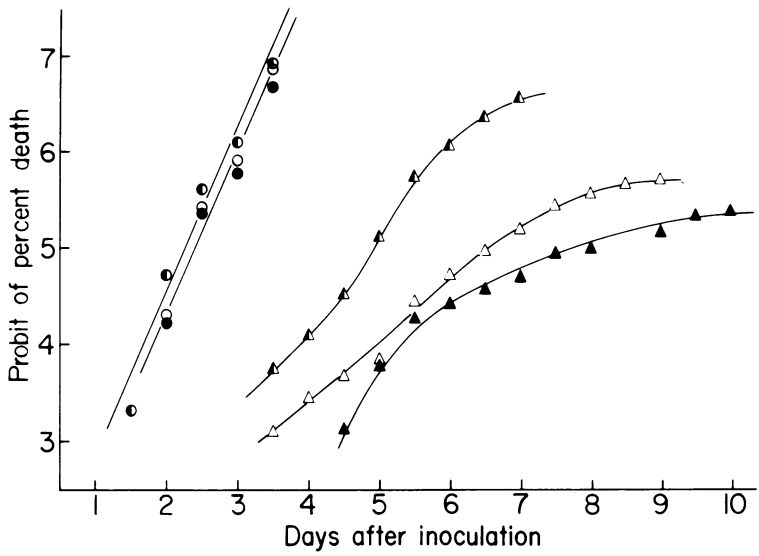


FIG. 1. *Cumulative death patterns of mice inoculated intracranially with the parent or the tsNG39 strain at various inoculum sizes. Fifteen to 30 adult male mice were intracranially inoculated with 10^7, 10^5, or 10^3 PFU of the PS or the tsNG39 strain. Clinical signs and death were observed every 12 h, and each cumulative curve was determined based on the results of three repeated experiments. Symbols: the PS—10^7 PFU (◐), 10^5 PFU (○), and 10^3 PFU (●); tsNG39—10^7 PFU (▲), 10^5 PFU (△), and 10^3 PFU (▲).*

injected with PS. The cumulative percent death curves of the ts-10^7, ts-10^5, and ts-10^3 groups were sigmoidal or logarithmic curvelike in shape on the probit scale, so they did not fit the cumulative frequency curve of the Gaussian distribution. The 50% death times of these three groups were about 5, 6.5, and 8 days, respectively, when read graphically. The final death rates, which were determined 10 days after injection, were 94.3, 77.4, and 65.5%, respectively. Consequently, the percent death and the final death rate seemed to vary depending on the tsNG39 inoculum size.

Virus growth in the mouse brain. To know the extent of growth of temperature-insensitive (TI) virus that formed plaques at nonpermissive temperature, plaque assays were done at 42 and 37 C, since the appearance of the TI virus seemed to be important in determining the death of mice that received tsNG39 in a previous experiment (14).

The virus titers in the mouse brain of the P-10^7, P-10^5, and P-10^3 groups augmented fast and reached the maximum within 2 days after inoculation (Fig. 2a, b, and c). The virus titers at 12 h after injection were different among these three groups and seemed to be dependent on the inoculum size, but their maximum titers obtained on day 2 were similar ($10^{7.6}$ to $10^{8.3}$ PFU/0.2 ml). In the all of these three groups, the virus titers assayed at 42 C (TI virus titers) showed the same time course with the titers assayed at 37 C (total virus titers), and little difference in PFU was found between them. The maximum titer was obtained at about the same time when one-half of the infected mice died. On the contrary, the total virus titers in the mouse brain injected with tsNG39 increased slowly and maximum titers ($10^{5.4}$ to $10^{6.1}$ PFU/0.2 ml) were attained 2 to 3 days after injection, decreasing gradually thereafter (Fig. 2d, e, and f). It is interesting that the time course of the TI virus titer varied remarkably depending on the initial PFU of injection. In the mice of the ts-10^7 group, the TI virus could detect on day 2 after inoculation, it did not detect before day 3 in the brain of the ts-10^5 group, and in the sample of the ts-10^3 group the TI virus was undetectable within the period of observation. Each 50% death time of these three groups was observed 2 to 5 days later than the time when the virus titer reached the maximum.

IF production in the mouse brain. It was reported that in brains of suckling mice the avirulent large plaque variant of Sindbis virus induced a larger amount of IF than the virulent small plaque variant until 24 h postinfection (13). To know if the reduced death rate of mice infected with tsNG39 was attributable to its superiority in IF production to PS, the IF yield in brains was examined in each group of the mice injected with PS or tsNG39. The time course of IF yield of each group showed almost

the same curve as the virus growth curve of the identical group (Fig. 3). No significant difference in the maximum titer of IF was observed among either the three PS-injected groups or the groups inoculated with tsNG39 at three different inoculum sizes, although the PS-injected groups showed higher IF titers than those of the groups injected with tsNG39. Moreover, tsNG39 had the same sensitivity in vitro to mouse IF as that of PS (data not shown).

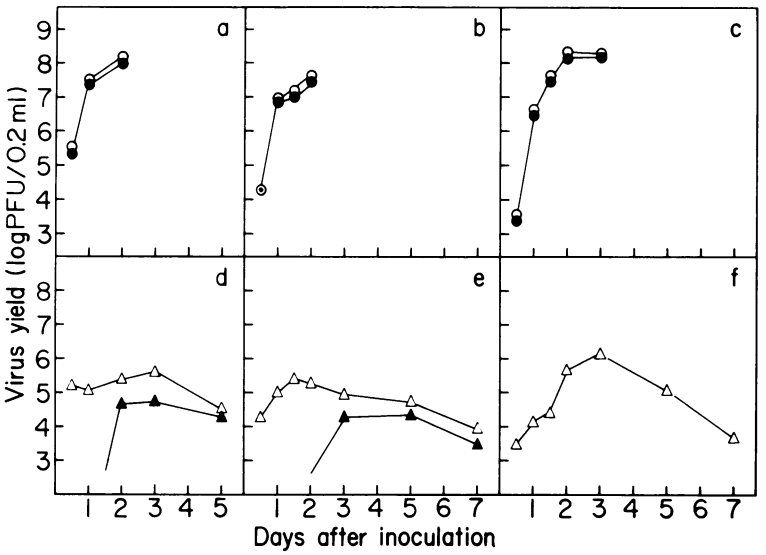


FIG. 2. *Virus growth in the mouse brain. Mice were inoculated with the parent or the tsNG39 strain at the inoculum size of 10^7, 10^5, or 10^3 PFU per brain. At the time indicated after injection, two mice were sacrificed at each point, and their brains were removed for virus assay. Plaque assay was performed at both 37 and 42 C. Open circles and triangles indicate the virus titer assayed at 37 C, and filled circles and triangles show the titers obtained at 42 C. (a, b, c) Virus titer in the mouse brain inoculated with 10^7 PFU (a), 10^5 PFU (b), and 10^3 PFU (c) of the PS. (d, e, f) Virus titers in the mouse brain injected with 10^7 PFU (d), 10^5 PFU (e), and 10^3 PFU (f) of the tsNG39 strain.*

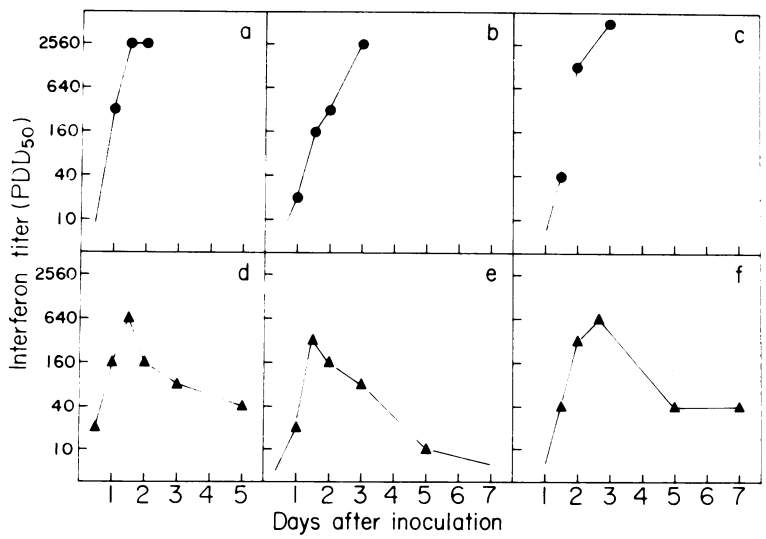


FIG. 3. *IF production in the mouse brain. At the selected time after the intracranial injection with the parent or the tsNG39 strain at the inoculum size of 10^7, 10^5, or 10^3 PFU, IF in the brain was assayed by the 50% plaque reduction method, and the titers were expressed by the 50% plaque-depressing dose. (a, b, c) IF titers in the brain injected with 10^7 PFU (a), 10^5 PFU (b), and 10^3 PFU (c) of the PS; (d, e, f) IF titers inoculated with 10^7 PFU (d), 10^5 PFU (e) and 10^3 PFU (f) of the tsNG39 strain.*

Histological changes of the mouse brain. (i) PS-injected mice. The most severe changes of the acute phase were observed in the brain of the P-10^7 group. The first changes appeared 24 h after injection, and they consisted of some vacuolar changes of ground substance which diffusely scattered in the hippocampus, thalamus, and corpus striatum (Fig. 5). At the same time, slight perivascular cuffing composed of only a few cells was found. Spongy degenerations, consisting of focal gathering of vacuoles, were found in the hippocampus, with slight pyknotic nerve cells. On day 3, these spongy changes occurred diffusely in the whole of the hippocampus and thalamus, and sometimes in the cortex, all with severe degenerating nerve cells. In these lesions, nerve cells as well as glia cells decreased in number (Fig. 6), and in some parts nerve cells disappeared completely. The lesions of the hippocampus were especially severe and were sharply demarcated from the surrounding tissue. Many perivascular cuffings were also seen in the cortex. The brain, after inoculation of 10^5 or 10^3 PFU of PS, exhibited spongy changes that were essentially the same pattern as those of the P-10^7 group, but the changes of both groups were not as severe as that of the P-10^7 group. On the other hand, the perivascular cuffings observed in the P-10^5 and P-10^3 groups were almost the same degree as that of the P-10^7 group (Fig. 4). Some small mononuclear cells were found in the submeningeal space, but the condition was far from being diagnosed as meningitis. Focal bleeding and pyknotic nerve cells were found around the site of injection.

(ii) tsNG39-injected mice. The spongy changes with degenerating nerve cells appeared in the thalamus only approximately 5 days after inoculation (Fig. 7). These changes appeared later and were slighter in degree than those of the PS-injected groups. Contrary to spongy changes, inflammatory ones, which consisted of perivascular cuffings and cell proliferations (Fig. 10), appeared from the early stage of infection and persisted in an increasing degree

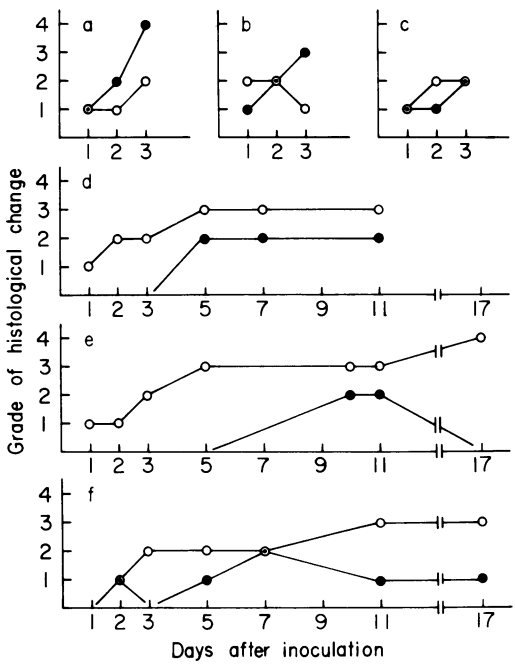


FIG. 4. *Time course of the histological changes in the mouse brain injected with the parent or the tsNG39 strain. Mouse brains were removed for histological examinations at the time indicated after injection with 10^7 PFU (a), 10^5 PFU (b), or 10^3 PFU (c) of the PS, or after inoculation of 10^7 PFU (d), 10^5 PFU (e), or 10^3 PFU (f) of the tsNG39 strain. Separate estimates were made for degenerative (●) and inflammatory (○) changes. Each of these changes was divided into four grades following the criteria described in Materials and Methods.*

(Fig. 4). At 10 days postinjection, moderate to severe perivascular cuffing was observed with glia cell proliferations throughout the brain (Fig. 8). The degenerative change, however, was not obvious, and a slight vacuolic change of ground substance was seen in only a few cases. The brains of two mice that died at 3.5 and 6.5 days after the injection of 10^5 PFU of tsNG39 showed more severe spongy changes than those of the sacrificed mice of the same group; i.e.,

FIG. 5. *Vacuolic change in the corpus striatum 2 days after injection of 10^7 PFU of the PS. Hematoxylin and eosin stain. ×250.*

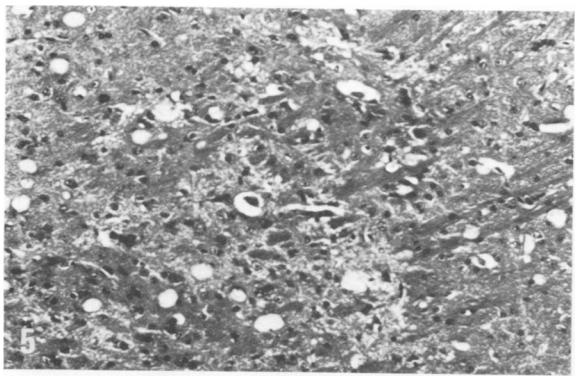
FIG. 6. *Severe spongy change with pyknotic nerve cells of the hippocampus 3 days after inoculation of 10^3 PFU of the PS. Hematoxylin and eosin stain. ×250.*

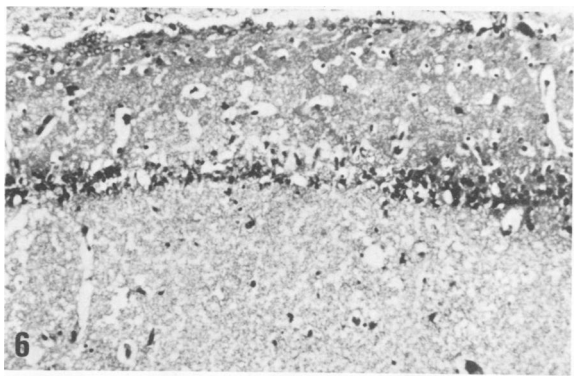
FIG. 7. *Perivascular cuffings with glia cell proliferation in the thalamus 5 days after injection of 10^7 PFU of the tsNG39 strain. Hematoxylin and eosin stain. ×250.*

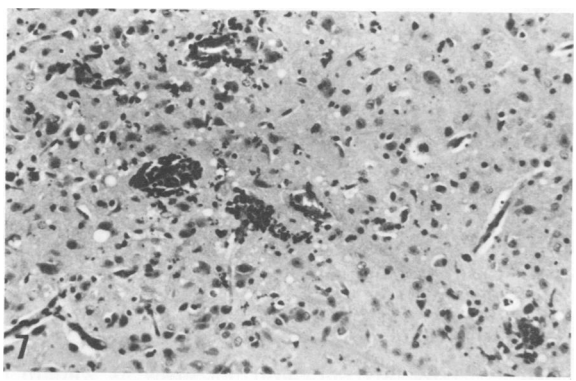
FIG. 8. *Moderate perivascular cuffing and pyknotic nerve cells in the hippocampus 10 days after injection of 10^3 PFU of the tsNG39 strain. Hematoxylin and eosin stain. ×250.*

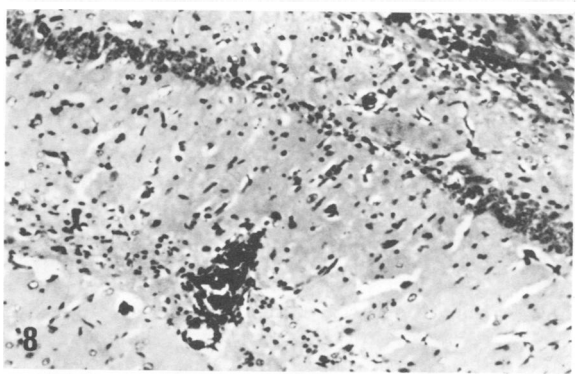
FIG. 9. *Severe degenerative change of the hippocampus with degenerative nerve cells and the loss of nerve cells in a mouse that died 3.5 days after injection of 10^5 PFU of the tsNG39 strain. Hematoxylin and eosin stain. ×250.*

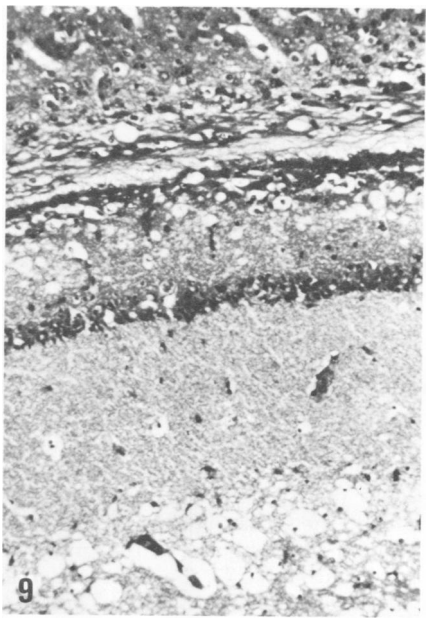
FIG. 10. *Moderate perivascular cuffing of the thalamus 5 days after injection of 10^7 PFU of the tsNG39 strain. Hematoxylin and eosin stain. ×250.*

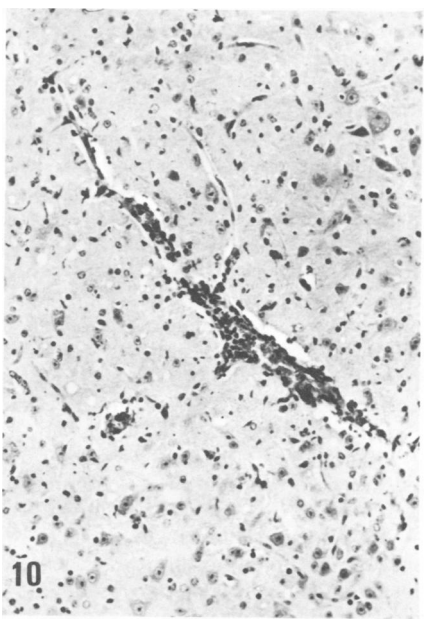












FIGS. 5–10

moderate to severe spongy changes were found in the whole of the hippocampus and slight perivascular cuffings were focally observed in the cortex of these dead mice (Fig. 9).

As a control, the brains injected with 0.02 ml of diluent or inoculated with the ultraviolet-inactivated PS or tsNG39 were examined 10 days after injection. They showed neither degenerative nor inflammatory change.

DISCUSSION

Marked differences in the cumulative death pattern were found between mice injected with PS and those inoculated with tsNG39. The PS-injected mice usually died at 2 to 3 days after inoculation when the virus yield attained the maximum, irrespective of the inoculum size of virus, whereas none of the mice injected with tsNG39 died within 3 days after inoculation, although the virus titer had already reached its maximum. Among the three groups of mice injected with tsNG39, the difference in lethality of mice was obvious (Fig. 1). The lethality of these mice seemed to be correlated with the initial inoculum size of virus rather than with the maximum titer in the brain. The number of mice dying from the infection of tsNG39 began to decrease from day 6 after injection. By this time the virus titer in the brain had already ceased to increase, as if some factors had forced the virus growth to decrease. IF does not appear to be a candidate for this, as stated by some authors (3, 5, 10), since the IF titer was found to change parallel to the growth curve of tsNG39 in the course of infection, and it was always lower than that in the mice injected with PS. It is presumably an immunological mechanism that worked in decreasing the number of mice that died from the infection of the attenuated virus, as already reported (1, 20).

By histological examination, a marked difference was also found between the mouse brains injected with PS and those inoculated with tsNG39. In these brains two types of reactions were distinguished, inflammatory and degenerative changes, as reported by King (8, 9). In the groups of PS-injected mice, the degenerative change was well correlated with the virus growth in the brain. On the other hand, in the tsNG39-injected groups the dominant change was inflammatory, and the slight-to-moderate degenerative changes appeared only in the later stage of infection (Fig. 4). However, the degenerative change was more severe in the dead mice than in the sacrificed mice after injection of tsNG39.

Contrary to the growth in vitro (14), the maximum titer of tsNG39 in the mouse brain was lower than that of PS by approximately $10^{2.4}$ PFU/0.2 ml, and the virus titers in the brain received tsNG39 always below 10^7 PFU/0.2 ml over the period of observation, although PS attained above 10^7 PFU within 1.5 days after inoculation. Since the degenerative changes resulted from the direct virus effect, namely, the disruption and dysfunction of cells (17), and because the inflammatory reaction was due to an immune reaction initiated by the infecting virus (4, 17, 20, 21), the difference in histology between the mice that received PS and those injected with tsNG39 reflected well the difference in the intracranial virus titer observed between these groups of mice. Consequently, it is estimated that tsNG39 did not grow as rapidly in the brain as PS did, so injuries of the brain cells extend slowly enough to allow the mice to live; we assume that a relatively low grade of virus growth in the brain permitted some of the mice to survive until their immunological defense became effective enough. The bizarre death pattern observed in a pervious experiment (14) was presumably due to individual differences in the immunological response of the mice.

The factor that plays the most important role in determining the death of the infected mice seems to be the velocity of the virus growth in the mouse brain and the maximum titer attained in a short period. The TI virus, which is thought to be a back mutant and appeared in the multiple growth cycles of tsNG39 in the brain, does not seem to play an important role in determining the death of mice, since the TI virus could not grow as much as PS, presumably because of the kind of interference with tsNG39 described by Zebovits and Brown (19). This is supported by the result that there was too little TI virus to be detected in our assay system when the mice were injected with 10^3 PFU of tsNG39.

Why tsNG39 could not grow as well in the mouse brain as in vitro remains unknown. The one possible explanation is as follows. tsNG39 could not grow as well in vivo as in vitro because it is sensitive to an elevated temperature and to a low acid pH due to a local inflammation, as stated by Lwoff and Lwoff (12) and as observed in the in vitro tests of another ts mutant of Western equine encephalitis virus (15). The other explanation is related to the mechanism by which the virus spreads and invades the brain tissue. After intracranial injection the virus particles seemed to spread mainly by blood stream and to invade the brain tissue through the endothelium of small vessels, as described by Johnson and Mims (7), because no

trace of viral encephalitis was found around the needle track and the histological changes were observed similarly in both cerebral lobes within a short period after injection. Provided that tsNG39 cleared from the blood stream faster than PS, as observed in Mengo virus (2) and Venezuelan encephalitis virus (6), the intracerebral titer of tsNG39 would be maintained at a lower level than that of PS even though tsNG39 produced the same amount of infectious particles in the brain as PS. The second explanation seems to be more likely than the first, although our results do not permit a determination of which is more likely.

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