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Induction of Demyelination by a Temperature-sensitive Mutant of the Coronavirus MHV-A59 Is Associated with Restriction of Viral Replication in the Brain

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SUMMARY

The neurovirulence of eight temperature-sensitive (*ts*) mutants of mouse hepatitis virus strain A59 in 4-week-old BALB/c mice was investigated. Whereas a dose of 100 p.f.u. of wild-type virus killed mice within a week, a 1000-fold higher dose of *ts* mutants did not. Three *ts* mutants induced demyelinating disease in the central nervous system (CNS). The pathology of the demyelinating disease caused by one mutant, designated *ts*-342, was studied in detail. Pathological changes, starting 3 days post-inoculation (p.i.), were characterized by inflammation and demyelination in the CNS. Antibody responses directed against all virus-specific structural proteins were present at 7 days p.i. No virus particles were observed by electron microscopy at 14 days p.i. However, macrophages and lymphocytes were abundant in the areas of demyelination. The growth kinetics *in vivo* of wild-type virus, *ts*-342 and a revertant of *ts*-342 were compared. Wild-type virus and the revertant replicated rapidly in the brain and spread to the liver causing a lethal hepatitis. *Ts*-342, however, replicated to a much lesser extent within the brain and could not be detected in the blood or liver. The *ts* lesion in the genome of *ts*-342 seems, therefore, to determine the outcome of the infection.

INTRODUCTION

Mouse hepatitis virus (MHV) belongs to a group of enveloped animal RNA viruses, the Coronaviridae (Tyrrell *et al.*, 1978; Siddell *et al.*, 1983). In rodents of the appropriate age and genetic background, intracerebral inoculation of a large dose of wild-type MHV causes an acute lethal disease (Knobler *et al.*, 1981, 1982; Wege *et al.*, 1982). Low doses of wild-type virus or high doses of attenuated temperature-sensitive (*ts*) mutants of MHV strains JHM and A59 produce a chronic demyelinating disease in mice (Haspel *et al.*, 1978; Koolen *et al.*, 1983; Lavi *et al.*, 1984*b*; Weiner, 1973; Woyciechowska *et al.*, 1984). Demyelination induced by MHV-JHM, which has been studied most extensively, is characterized by infection and lysis of oligodendrocytes, leaving axons well preserved (Sorensen *et al.*, 1980). Similar lesions develop in C57BL/6 mice surviving an initial infection with MHV-A59 wild-type virus (Lavi *et al.*, 1984*b*). In weanling C3H mice which are genetically resistant to MHV, intracerebral inoculation of MHV-A59 wild-type virus causes a demyelinating disease in which viral antigens can be detected in oligodendrocytes for up to 4 weeks post-inoculation (p.i.) (Woyciechowska *et al.*, 1984). Lavi *et al.* (1984*a*) showed that although no infectious virus was detectable and only low

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levels of viral antigen were present in C57BL/6 mice intracerebrally infected with MHV-A59, virus-specific RNA could still be detected in the central nervous system (CNS) and liver 10 months p.i. In BALB/c mice, MHV-A59 causes, even at low dosage, an acute infection with high mortality (Koolen *et al.*, 1983).

Intracerebral inoculation of certain *ts* mutants of MHV-A59 causes a subacute demyelinating disease in the CNS (Koolen *et al.*, 1983). The present paper describes virological and neuropathological findings in mice infected with *ts* mutants of MHV-A59, including several that induce demyelination in the CNS. One of these mutants, *ts*-342, was subjected to more detailed sequential neuropathological study. The results of these studies suggest a possible explanation for the differences in the neurovirulence of wild-type virus and *ts*-342.

METHODS

Virus and animals. The isolation and characterization of the *ts* mutants of MHV-A59 and the preparation of virus stocks were described previously (Koolen *et al.*, 1983), as was the isolation of revertants of *ts*-342 (van Berlo *et al.*, 1986). The ratio of *ts*-342 plaque titres at 40 °C to those at 31 °C was about 10^{-5}, compared with ratios of 1·5 to 8 for the revertants or wild-type virus.

Four-week-old BALB/c mice (Centraal Proefdierenbedrijf TNO, Zeist, The Netherlands) were used. Before infection, animals were bled and their sera checked in a plaque reduction assay, to ensure the absence of anti-MHV antibodies. Groups of four mice were injected intracerebrally with 10^2, 10^3 or 10^5 p.f.u. of each virus in 0·1 ml phosphate-buffered saline (PBS). Control animals received PBS without virus. Groups of animals injected with different doses of *ts* mutants were kept in separate cages in the same isolator. The virus did not spread to sentinel animals kept in the same isolator. The mice were monitored daily for clinical signs of neurological disease and were sacrificed 4 weeks p.i.

Thirty-six mice were infected with 10^5 p.f.u. of *ts*-342. After 0, 3, 7, 10, 14, 17, 21, 24, 28, 42, 56 or 112 days, mice were anaesthetized with ether and perfused through the left ventricle with paraformaldehyde (4% w/v in 0·2 M-phosphate buffer pH 7·0) for paraffin histology, or with paraformaldehyde (0·5% w/v) and glutaraldehyde (4% w/v) in 0·1 M-cacodylate buffer pH 7·0 for electron microscopy (Moolenbeek, 1982). Tissue for histology was embedded in paraffin wax and 5 μm sections were stained with haematoxylin and eosin. Tissue for electron microscopy was post-fixed in 1% osmium tetroxide, dehydrated in graded alcohols and embedded in a mixture of Epon and Araldite. Sections 1 μm thick were stained with toluidine blue or paraphenylenediamine and ultrathin sections with uranyl acetate and lead citrate.

Spread of virus after intracerebral infection. Groups of eight mice were infected intracerebrally with 10^5 p.f.u. of virus in 0·1 ml PBS. At 4, 8, 16 and 24 h p.i., two mice from each group were sacrificed and frozen sections of brain and liver were examined by immunofluorescence for the presence of viral antigen (see below). Heparinized samples of blood taken just before sacrifice were either centrifuged at 1000 *g* or frozen and thawed twice. The titres of infectious virus in the plasma and in the frozen and thawed blood samples were determined by plaque titration on monolayer cultures of L-cells (Koolen *et al.*, 1983).

The kinetics of viral growth in the CNS were determined by plaque titration of virus isolated from the infected brains. Groups of 12 mice were infected intracerebrally with 10^5 p.f.u. of virus. At the times indicated above, each of three infected brains was removed and homogenized by trituration in 1 ml Dulbecco's MEM supplemented with 3% foetal calf serum. After centrifugation of the homogenate for 10 min at 10000 *g*, the supernatants were plaque-titrated on monolayer cultures of L cells as previously described (Koolen *et al.*, 1983).

Immunofluorescence of frozen sections. The brains and livers of infected mice were frozen in isopentane. Sections were cut at 4 μm, fixed at −20 °C for 10 min in 5% acetic acid in ethanol, washed with PBS and incubated for 1 h with a fluorescein isothiocyanate-conjugated IgG fraction of rabbit anti-MHV-A59 hyperimmune serum (Rottier *et al.*, 1981*b*), diluted 1 : 200 in PBS supplemented with 5% bovine serum. After incubation, sections were washed with PBS, mounted in 90% glycerol in PBS pH 9·0 to which 10 μg/ml phenylenediamine had been added to reduce photobleaching (Johnson & Nogueira Araujo, 1981), and examined for immunofluorescence.

Assay for antiviral antibodies in infected mice. Viral proteins were labelled as described previously (Koolen *et al.*, 1984). Briefly, 25 cm^2 monolayer cultures of Sac(−) cells (6 × 10^6 cells) were infected with 50 p.f.u./cell of wild-type virus or mock-infected. Viral proteins were labelled from 7 to 9 h p.i. with 60 μCi of L-[^{35}S]methionine (1420 Ci/mol; Amersham) in 2·5 ml of methionine-deficient MEM supplemented with 5% foetal calf serum. After labelling, cells were lysed in 0·5 ml lysis buffer (0·02 M-Tris–HCl, 1 mM-EDTA, 0·1 M-NaCl, 0·5% Triton X-100, 0·5% 1,5-naphthalene disulphonate. Na_2 pH 7·4) and a cytoplasmic fraction was obtained by centrifugation for 5 min at 10000 *g*. The immunoprecipitation reaction mixtures, containing 5 μl cell lysate, 600 μl lysis buffer and 10 μl of serum taken at various times after infection with *ts*-342 were incubated overnight at 4 °C. After addition of 1 μl rabbit anti-mouse IgM (Fc) (Nordic Pharmaceuticals, Tilburg, The Netherlands), incubation was continued for 4 h at 4 °C. The immune complexes were adsorbed to *Staphylococcus aureus* (Kessler, 1975; Koolen *et al.*, 1983)

and virus-specific proteins were analysed on 15% SDS–polyacrylamide gels (Rottier *et al.*, 1981*a*).

Virus-neutralizing antibodies were quantified by plaque reduction assay. Serum was diluted with PBS supplemented with 1% foetal calf serum (PBS–1%FCS) and then mixed with an equal volume (300 µl) of PBS–1%FCS containing 300 p.f.u. of virus. Hyperimmune mouse serum diluted with PBS–1%FCS, and PBS–1%FCS alone, served as controls. Antibodies were allowed to react with the virus for 1 h at 37 °C. Monolayer cultures of L cells (2×10^6 cells/well) were infected as described previously (Koolen *et al.*, 1983). One day after infection, plaques were counted and antibody titres determined. These were expressed as the reciprocal of the serum dilution that neutralized 50% of the virus (Reed & Muench, 1938).

RESULTS

Neurovirulence of wild-type virus and ts *mutants*

Intracerebral infection of 4-week-old BALB/c mice with 10^2 or 10^5 p.f.u. of MHV-A59 wild-type virus was lethal within 5 and 2 days respectively (Table 1). Histological examination revealed acute panencephalitis and hepatitis. On electron microscopic examination of the brain stem of mice infected with 10^5 p.f.u. of wild-type virus, virus particles in various stages of maturation were observed in the leptomeninges and in astrocytes (Fig. 1*a* and *b*).

When 10^3 or 10^5 p.f.u. of any of the eight *ts* mutants were injected intracerebrally into mice, no clinically obvious disease developed, except in the case of *ts*-169 and *ts*-342 at a dose of 10^5 p.f.u. (Table 1). *Ts*-169 infected animals showed ruffled fur and hunching of the spine 7 to 10 days after infection. One *ts*-169-infected mouse was sacrificed 7 days p.i. and the brain, spinal cord, liver, spleen and kidneys were examined histologically. Only in the CNS were changes observed. These consisted of multiple foci of necrosis and inflammation, which affected the grey and white matter and the ependyma. The other *ts*-169-infected mice recovered and appeared clinically normal at the end of the experiment (4 weeks after infection).

Mice infected with the mutant *ts*-342 showed dragging of their hind limbs and abnormal righting behaviour at 2 days p.i. All six animals manifested abnormal positional nystagmus starting at day 2 and persisting at least 6 months (Koolen *et al.*, 1985). Histological abnormalities were determined at 4 weeks p.i. and were present in the CNS of mice infected with *ts*-43, *ts*-169 and *ts*-342. The caudal brain stem and spinal cord showed vacuolar disruption of the white matter (Fig. 2). Perivascular and leptomeningeal infiltration by mononuclear inflammatory cells was quite often observed (data not shown). Such lesions were present in one of four mice infected with 10^5 p.f.u. of *ts*-43, in all three mice infected with 10^5 p.f.u. of *ts*-169 and in all mice infected with 10^3 or 10^5 p.f.u. of *ts*-342. The animals infected with 10^5 p.f.u. of *ts*-201, *ts*-209 or *ts*-379 but not those infected with *ts*-299 produced virus-neutralizing antibody with titres of 130 to 1000 at 4 weeks p.i. (Table 1).

The ability of the mutants to induce pathological changes in the CNS was examined and compared to the biological and biochemical alterations induced by these mutants in tissue culture cells (Table 1). Mutants of three different phenotype classes (RNA^-/$protein^-$, RNA^+/$protein^-$ and RNA^+/$protein^+$) were included in the eight tested; no correlation was found between the biochemical alterations caused by mutants *in vitro* and their neurotropism *in vivo*.

Sequential study of neuropathology after infection with ts-*342*

Ts-342, at a dose of 10^5 p.f.u. was chosen for study of the neuropathology in more detail. We wanted to determine: (i) how soon after infection lesions developed in the CNS; (ii) whether or not the difference in pathology compared to that of the wild-type virus was due to a single *ts* mutation; (iii) whether viral antigens were expressed in the CNS; and (iv) whether infection caused production of antiviral antibodies.

Time course of ts-*342-induced neuropathology*

At 3 days p.i. there were many small foci of necrosis in the grey and white matter. Scattered lesions were present throughout the CNS and were particularly numerous in the brain stem and spinal cord. These lesions were associated with scanty perivascular and leptomeningeal accumulation of neutrophils and mononuclear cells (Fig. 3*a*). Scattered neutrophils had

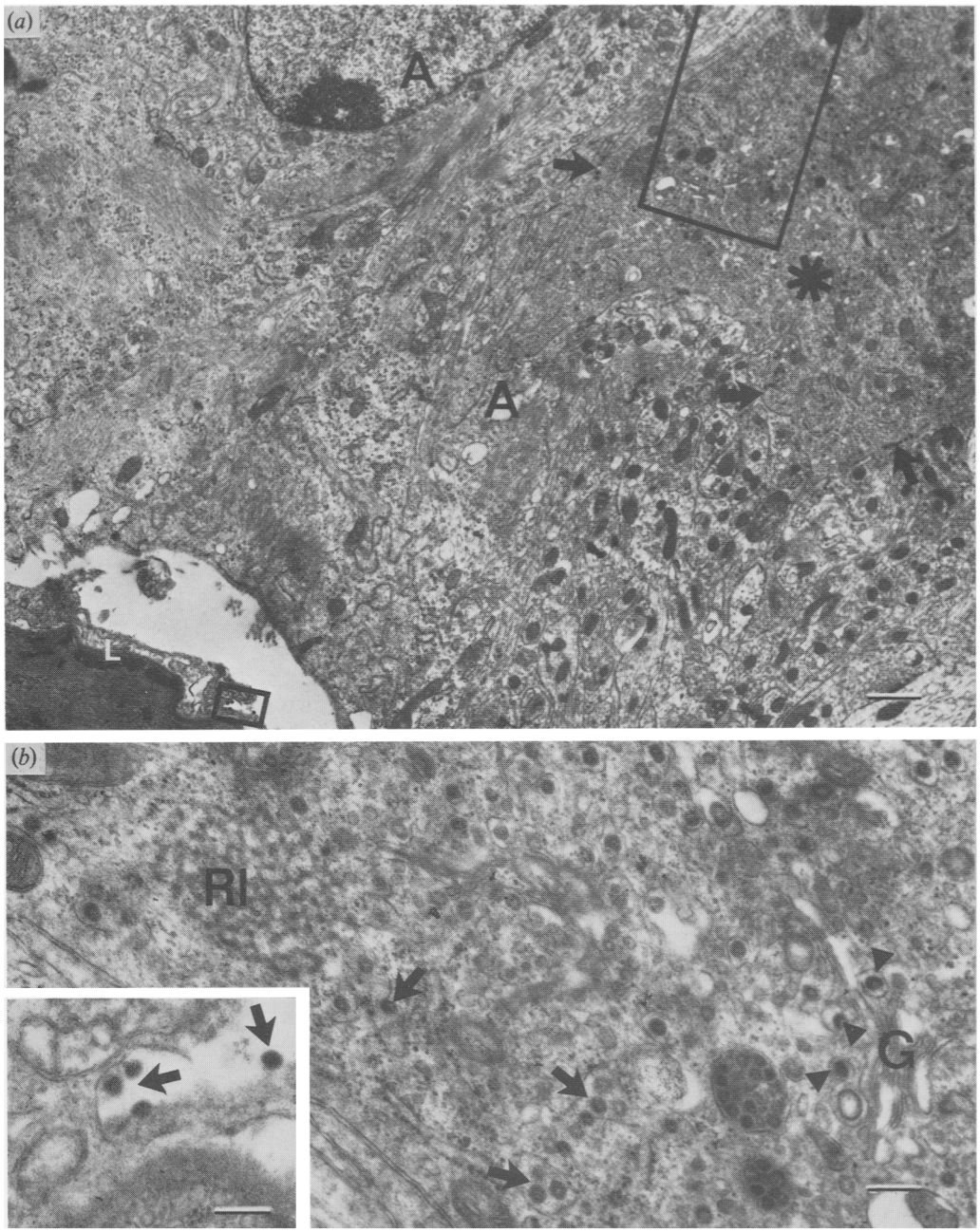


Fig. 1. Electron micrographs showing a section of the brain stem from a BALB/c mouse, 2 days after infection with wild-type virus. (*a*) Low magnification showing part of the ventral surface with adjacent leptomeninges (L). Astrocytes (A) surround a cell (asterisk) with abundant virions in the cytoplasm and in the extracellular space (arrows). (*b*) Higher magnification of the area marked in (*a*). Budding and free virions are seen in vesicles (arrowheads) associated with the Golgi apparatus (G), and in cisternae of the rough endoplasmic reticulum (arrows). RI, a tubuloreticular inclusion. Inset, extracellular virus particles (arrows) in the leptomeninges. Bar markers represent (*a*) 1 μm, (*b*) 250 nm and inset, 200 nm.

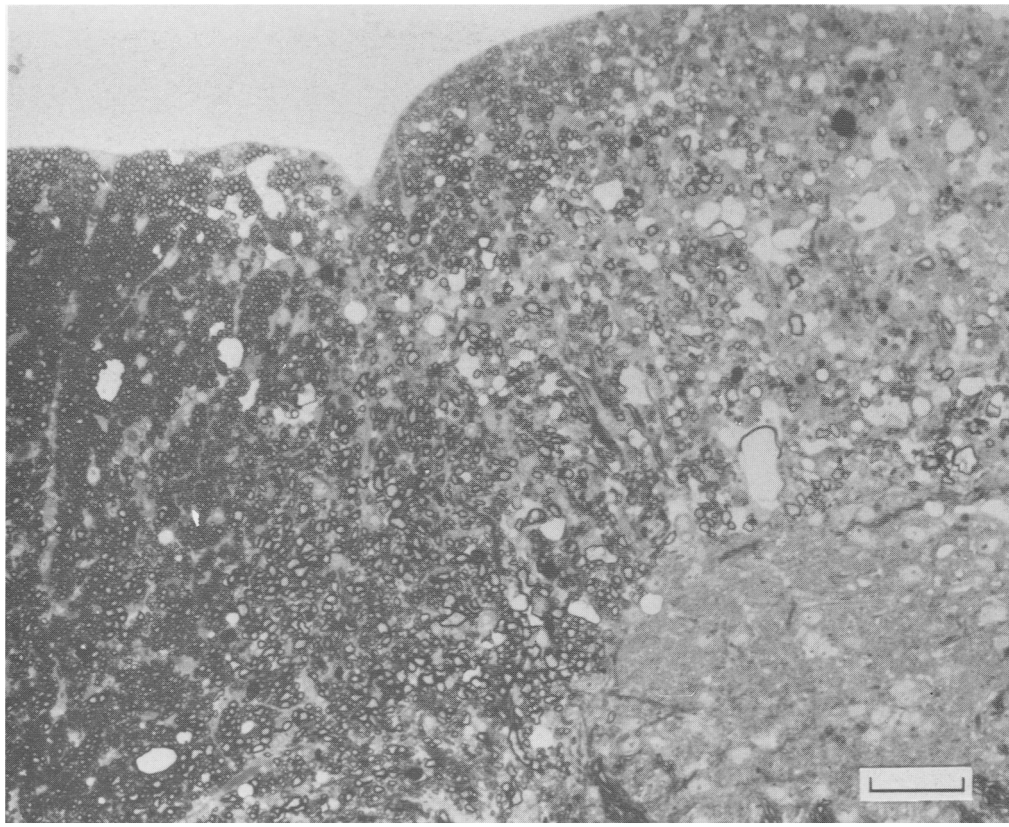


Fig. 2. Transverse section through the spinal cord 4 weeks after intracerebral infection with 10^5 p.f.u. of *ts*-342. There is extensive demyelination and some axonal degeneration in one dorsal column. Paraphenylenediamine staining was used. Bar marker represents 25 μm.

infiltrated the parenchyma. Lesions were especially numerous immediately adjacent to the lateral ventricles. There was patchy denudation of the ventricular lining and some haemorrhage and accumulation of inflammatory cells in the ventricles.

At 7 days after infection the cellular response was predominantly mononuclear. Lesions were most conspicuous in the white matter, where vacuolation of myelin and some axonal degeneration caused a moth-eaten appearance (Fig. 3*b*).

At later stages (10 to 112 days p.i.) the lesions were almost exclusively confined to the white matter (Fig. 3*c*). The cellular response gradually subsided during the first few weeks. Occasional lipid phagocytes were present and there was marked fibrillary gliosis (Fig. 3*d*).

Electron microscopic examination of lesions in the spinal cords of mice sacrificed 2 weeks after infection showed many demyelinated axons and a few fibres which had been thinly re-myelinated by oligodendrocytes (Fig. 4). Numerous macrophages containing myelin debris were present within the lesions. Viral particles were not observed.

In only two of six mice, one sacrificed at day 42 and the other at day 56 after infection, were no lesions found. Several mice, which developed slight to moderate hydrocephalus, showed granular ependymitis and subependymal gliosis. The site of injection was often identifiable as a slit-like scar with haemosiderin-laden macrophages.

Due to the ts *lesion* ts-*342 does not spread from the CNS*

Mice were infected intracerebrally with wild-type virus or *ts*-342 and the kinetics of virus growth in the brain were determined during the first 24 h following infection. In brains of mice

Table 1. *Relationship of neuropathology to antibody response and growth* in vitro *of eight* ts *mutants of MHV-A59*

Virus	RNA/protein* synthesis at 40 °C	P.f.u. inoculated†/ no. of mice	Mortality‡/ no. of mice	Neuropathology	Antibody§ titre	Progeny* yield (p.f.u./ml) 37/31 °C	E.o.p.* 40/31 °C
Wild-type	+/+	10^2/5	5/5	Acute	–	0·600	1
		10^5/4	3/3	panencephalitis			
Ts-43	+/−	10^3/4	0/4	None	198	0·016	2×10^{-5}
		10^5/4	0/4	Demyelination	363		
Ts-169	−/−	10^3/4	0/3	None	843	0·180	3×10^{-3}
		10^5/4	0/3	Demyelination	579		
Ts-201	+/+	10^3/4	0/4	None	<30	0·089	$2{\cdot}5 \times 10^{-4}$
		10^5/4	0/3	None	1006		
Ts-209	+/+	10^3/4	0/3	None	<30	0·500	1×10^{-3}
		10^5/4	0/4	None	132		
Ts-276	−/−	10^3/4	0/4	None	<30	0·041	3×10^{-4}
		10^5/4	0/2	None	1025		
Ts-299	−/−	10^3/4	0/2	None	<30	0·001	$<2{\cdot}2 \times 10^{-7}$
		10^5/4	0/3	None	34		
Ts-342	−/−	10^3/4	0/4	Demyelination	1397	1·000	4×10^{-6}
		10^5/4	0/4	Demyelination	952		
Ts-379	+/+	10^3/4	0/3	None	63	0·001	$<6{\cdot}6 \times 10^{-6}$
		10^5/4	0/3	None	188		
Sentinel		PBS/21	0/19	None	<30		

* Koolen *et al.* (1983).
† Intracerebrally in 0·1 ml PBS.
‡ Mortality of mice started on day 2.
§ As determined in a plaque reduction assay at 4 weeks p.i.

infected with wild-type virus, titres increased to 10^4 to 10^5 p.f.u./brain (Fig. 5*a*). A 100- to 1000-fold lower amount of infectious virus was isolated from mice infected with *ts*-342, indicating that its replication was restricted in the CNS. The *ts* mutation itself seemed to be responsible for the restriction of replication, since revertant virus which was no longer *ts* had the same growth kinetics as wild-type virus.

To determine whether wild-type virus and *ts*-342 spread outside the CNS, virus titres in the blood were determined during the 24 h following infection. No virus could be detected after infection with *ts*-342 but circulating virus was found from 8 h after infection with either wild-type or revertant virus (Fig. 5*b*). At 24 h, viral antigen was detected in the brains of mice infected with wild-type virus, revertant virus or *ts*-342. Antigen was also present in the livers of mice infected with wild-type virus but not in those of mice infected with *ts*-342 (Fig. 5 and 6). Thus, unlike wild-type virus, *ts*-342 seemed not to spread outside the CNS. Wild-type virus and revertant virus were present in the blood of infected mice but not in the plasma, indicating that infectious virus was cell-associated.

In contrast to *ts*-342, revertant virus showed identical neurovirulence to wild-type virus. Mice intracerebrally infected with 10^5 p.f.u. revertant virus died 2 days p.i. A dose of 100 p.f.u. of three different revertants of *ts*-342 was lethal within 5 to 6 days. Revertant virus did spread from the brain to the liver, where there were foci of necrosis containing viral antigen.

Viral antigens in the CNS at different times after infection

Mice were infected intracerebrally with wild-type virus or *ts*-342 and the distribution of viral antigens during the first week after infection was determined by immunofluorescence. During the first 48 h following infection, the distribution of viral antigen of both wild-type virus and *ts*-342 was similar. Viral antigen was present in the meninges, ependyma and occasional areas of white matter (Fig. 6). Mice infected with wild-type virus did not survive the infection for more than 2 days. At 3 to 5 days after infection with *ts*-342 the number of viral antigen-containing cells in the white matter had increased, presumably due to local replication and spread of the virus.

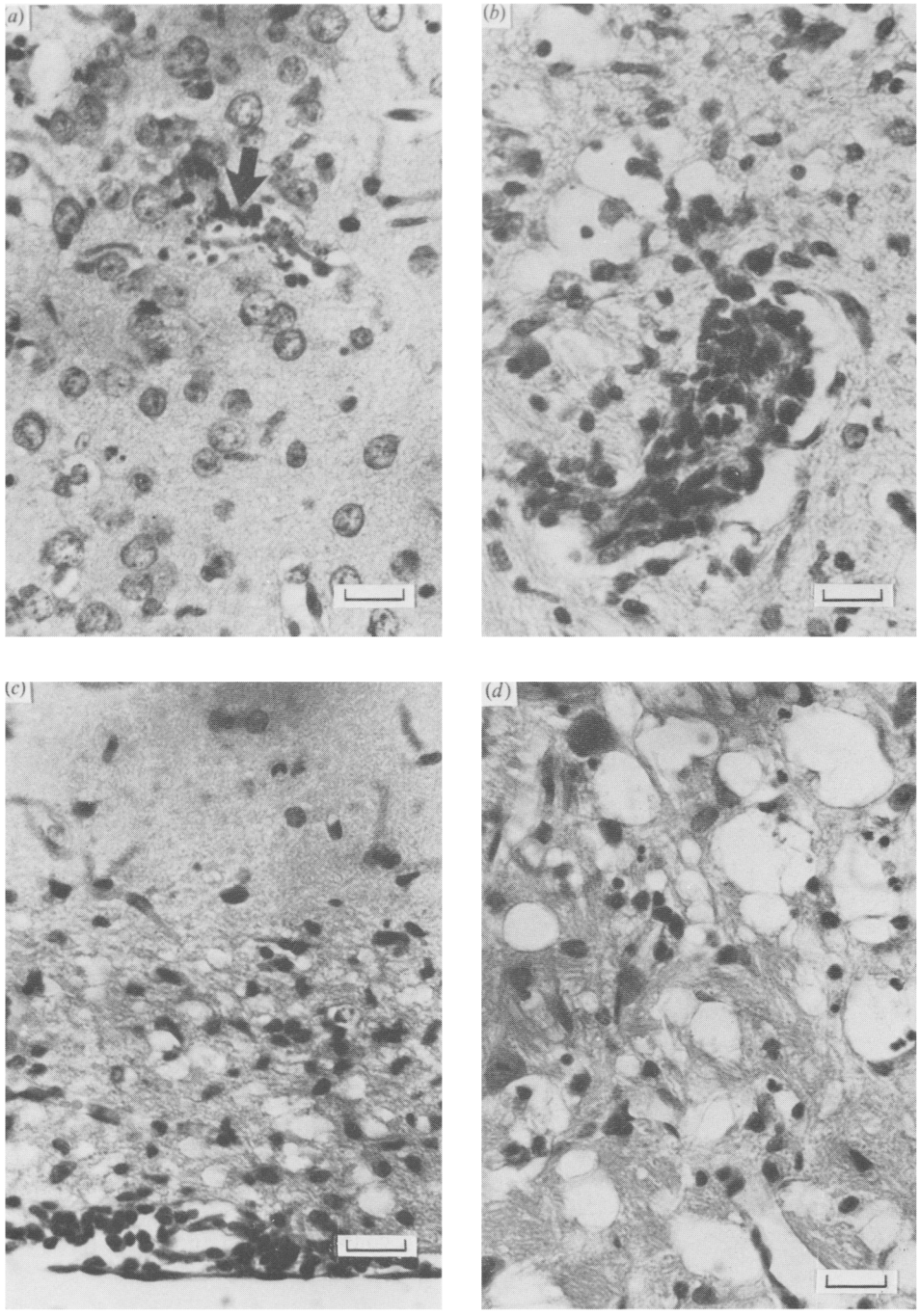
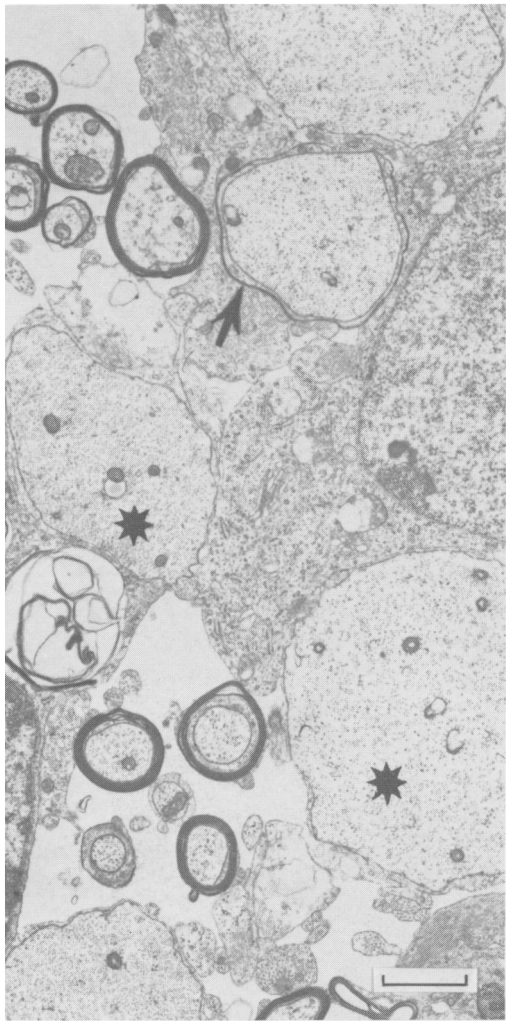


Fig. 3. Neuropathological changes in BALB/c mice intracerebrally infected with 10^5 p.f.u. of *ts*-342 at various times after infection. (*a*) Day 3. Cerebral cortex. Focus of necrosis with infiltration by inflammatory cells. (*b*) Day 7. Hypothalamus showing vacuolation, infiltration by mononuclear phagocytes, perivascular cuff. (*c*) Day 10. Olfactory tract. There is vacuolation of the white matter and infiltration of the leptomeninges and the neuropil by mononuclear cells. (*d*) Day 21. Tegmentum of the medulla, showing vacuolation and marked gliosis. Bar markers represent 50 μm.



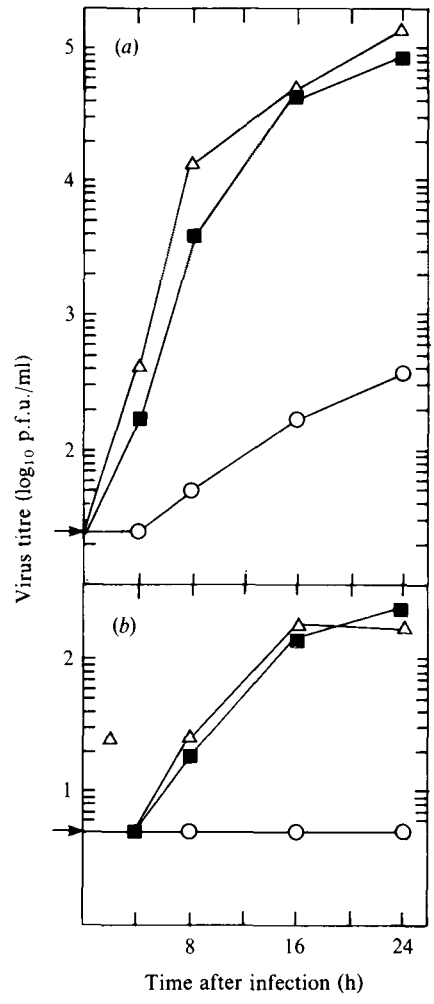


Fig. 5

Fig. 4. Electron micrograph of the ventral white matter of the spinal cord, 2 weeks after infection with *ts*-342 virus, showing several demyelinated axons (asterisks) and one thinly re-myelinated fibre (arrow). Bar marker represents 150 nm.

Fig. 5. Viral titres in (*a*) brains and (*b*) frozen and thawed blood samples of BALB/c mice, intracerebrally infected with 10^5 p.f.u. of wild-type virus (△), *ts*-342 (○), or revertant virus (■). Viral titres in the blood of mice infected with *ts*-342 were below the detection limit of the assay (arrow). Each point in (*a*) represents the mean of titres in three mice and each point in (*b*) the mean of titres in two mice.

Viral antigen was no longer detectable in the meninges and ependyma. At 6 days and later after infection, fewer antigen-containing cells (about one such cell in four to six spinal cord or brain sections) were observed and all were in the white matter (data not shown). Infected cells showed degenerative changes.

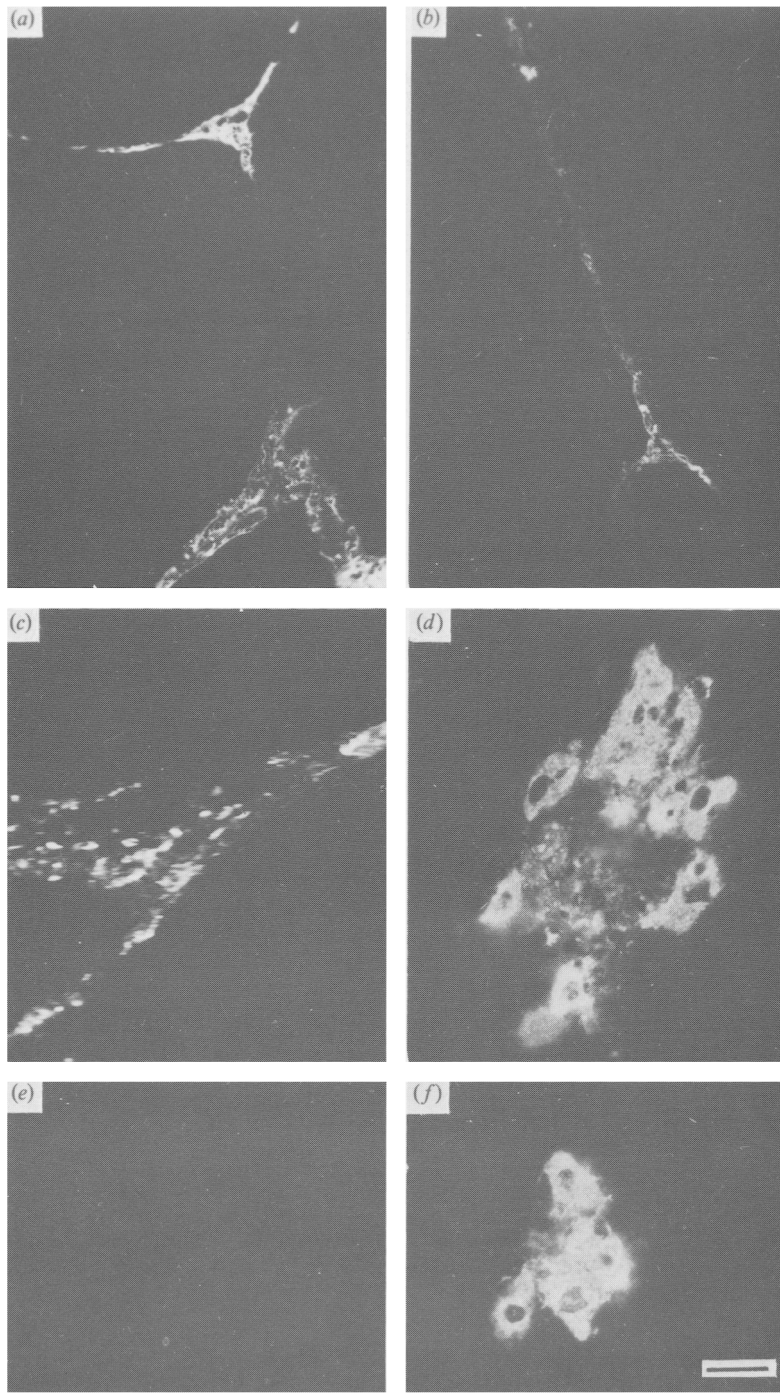


Fig. 6. Viral antigen, demonstrated by immunofluorescence, in frozen sections of brain (*a* to *c*) and liver (*d* to *f*), 24 h after intracerebral infection with 10^5 p.f.u. of wild-type virus (*a, d*), *ts*-342 (*b, e*) or a revertant of *ts*-342 (*c, f*). Bar marker represents 50 μm.

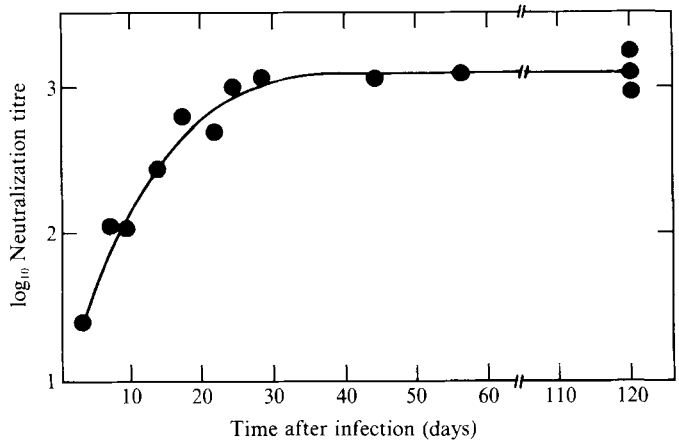


Fig. 7. Virus neutralizing antibody response at various times after intracerebral infection with 10^5 p.f.u. of *ts*-342.

Antibody production: kinetics and specificity for virion proteins

Serum samples, taken at various times after infection, were tested by plaque reduction assay. Antibody was detectable 3 days p.i. (Fig. 7). Twenty-four days p.i., antibody titres reached their maximum level and remained constant until the end of the experiment, at 112 days.

Sera tested in an immunoprecipitation assay revealed that in all cases, all virus-specific intracellular precursors for the three major viral proteins (Rottier *et al.*, 1981*b*; Sturman, 1977) were precipitated (data not shown).

DISCUSSION

This paper describes the neuropathology of infection with MHV-A59 wild-type virus and eight *ts* mutants in 4-week-old BALB/c mice. Although intracerebral inoculation of 10^2 p.f.u. wild-type virus was lethal within a week, inoculation of 1000-fold higher doses of the *ts* mutants was not. Three of these mutants caused demyelination within the CNS. The ability of different *ts* mutants to cause demyelination *in vivo* could not be correlated with any biochemical alterations caused in tissue culture cells *in vitro*. There was also no relationship between the induction of virus-specific antibodies and the ability of the mutants to induce neuropathological changes.

Recently, Buchmeier *et al.* (1984) showed that in mice infected with the neurotropic strain MHV-4 (JHM), antibodies may play an important role in the development of acute and/or chronic CNS disease. In MHV-4-infected mice, passive transfer of specific neutralizing monoclonal antibodies that recognize specific epitopes on the viral glycoprotein E2 prevented fatal infection by wild-type virus, which instead caused a non-lethal, chronic demyelinating disease. The antibodies seemed to block the infection of neurons but not of oligodendrocytes. More recently Dalziel *et al.* (1986) and Fleming *et al.* (1986) isolated variants of MHV-4 with mutations in the E2 peplomer glycoprotein that conferred resistance to neutralization by monoclonal antibodies. As a result of these mutations, variant virus caused a subacute demyelinating disease rather than acute encephalomyelitis. Haspel *et al.* (1978) reported that a specific *ts* mutant of the neurotropic strain MHV-4, designated *ts*-8, caused a high incidence of demyelination with low mortality. Pathological changes induced by *ts*-8 were characterized by selective destruction of oligodendrocytes (Knobler *et al.*, 1982). *In vitro* and *in vivo* studies of the neurohepatotropic strain MHV-A59 wild-type virus revealed that non-neuronal cells of the CNS are preferentially infected by the virus (Dubois-Dalcq *et al.*, 1982; Woyciechowska *et al.*, 1984). Apart from the first few days after *ts*-342 infection, viral antigen was confined to the white matter. However these results do not exclude a role for virus-specific antibodies in the pathogenesis of the demyelinating disease.

Recently, we showed that replication of *ts*-342 in cultured astrocytes from mouse brain was restricted at 37 °C, the presumed body temperature of the mouse. Nevertheless, *ts*-342 replicated well at 37 °C in Sac(−) cells in tissue culture (Koolen *et al.*, 1983; van Berlo *et al.*, 1986). The restricted replication of *ts*-342 in astrocytes *in vitro* presumably results from an interaction between the *ts* mutation of the virus and host-specific factors. The present findings indicate that replication of *ts*-342 is restricted in the brain *in vivo*. Since revertant viruses had identical growth kinetics and neurovirulence to those of wild-type virus, the *ts* mutation seems itself to determine the restricted replication and attenuated virulence. Whereas attenuation of neurovirulence of *ts*-342 is caused by a single *ts* mutation in the viral genome, the neurotropism of *ts*-8 is not. Revertant viruses of *ts*-8 do not regain the properties of MHV-4 wild-type virus, indicating that the attenuation of neurovirulence is not due solely to the *ts* mutation (M. J. Buchmeier, personal communication). The induction of subacute demyelinating disease by *ts*-342 is probably dependent on the inability of the mutant virus to spread from the brain and cause hepatitis. Fulminant hepatitis seems to be the cause of death of mice infected with wild-type virus. The restricted replication of *ts*-342 in astrocytes *in vitro* is in keeping with the restricted replication in the brain, but it has not been established whether *ts*-342 infects astrocytes *in vivo*. The presence of inflammatory cells in *ts*-342-induced lesions and the sparsity of viral antigen after the first few days of infection suggest that the demyelination may not be solely due to cytolytic infection of oligodendrocytes. Immunological factors may also be involved. Watanabe *et al.* (1983) have shown that adoptive transfer of T-cells from MHV-infected rats and re-stimulated *in vitro* with myelin basic protein, causes experimental allergic encephalomyelitis-like inflammation in the CNS of recipients. However, there is as yet no evidence that demyelinating disease can be produced by adoptive transfer of lymphocytes from MHV-infected mice.

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