Proc. Natl. Acad. Sci. USA
Vol. 75, No. 8, pp. 4033–4036, August 1978
Neurobiology

Temperature-sensitive mutants of mouse hepatitis virus produce a high incidence of demyelination

(coronavirus/model system/electron microscopy/virus virulence)

MARTIN V. HASPEL*†, PETER W. LAMPERT‡, AND MICHAEL B. A. OLDSTONE*

* Department of Immunopathology, Scripps Clinic and Research Foundation, La Jolla, California 92037; and ‡ Department of Pathology, School of Medicine, University of California, San Diego, La Jolla, California 92093

Communicated by Frank J. Dixon, May 22, 1978

ABSTRACT **Mutagenesis of mouse hepatitis virus with 5-azacytidine or 5-fluorouracil yielded several temperature-sensitive mutants. Mutants have been isolated that dramatically enhance the production of demyelinating disease over that previously noted with the wild-type virus. This reproducible model should now make possible the precise elucidation of the pathogenic mechanism and molecular basis of this virus-induced demyelination.**

A virus etiology has been proposed for several human demyelinating diseases, including multiple sclerosis (1). Studies of the pathogenic mechanism and molecular biology of virus-induced demyelination have been hampered by the unavailability of a feasible and reproducible animal model having a high incidence of demyelination. Mouse hepatitis virus (MHV) type 4 (JHM), a coronavirus, normally produces a rapidly fatal encephalomyelitis (2, 3). Under some conditions, such as low virus dose, MHV can, somewhat unpredictably, produce nonfatal demyelination due to a selective destruction of the myelin-synthesizing oligodendrocytes (4, 5). The early events in the pathogenesis of wild-type MHV-induced demyelination were difficult to evaluate because most of the infected animals either succumbed to the fatal encephalomyelitis or did not develop demyelination. Because selection for temperature-sensitive (ts) mutants has been shown by workers in our laboratory and different laboratories to often result in an alteration in the pathogenesis of other wild-type virus diseases (6–9), we sought, through the use of ts mutants, to attenuate MHV and thereby shift the disease spectrum from the fatal acute disease to the demyelinative disorder. We report the establishment of a reproducible model for the study of virus-induced demyelination.

MATERIALS AND METHODS

Virus Assay. Mouse hepatitis virus type 4 (JHM), kindly provided by L. Weiner (University of Southern California) was plaque purified in NCTC-1469 cells (10). The infectious titer, expressed as plaque-forming units per ml (PFU/ml), was determined by plaque assay on NCTC-1469 cell monolayers after 48 hr at 34°.

Virus Mutagenesis. The virus was grown overnight in NCTC-1469 cells at 34°C in the presence of 5-azacytidine (12 μg/ml) or 5-fluorouracil (150 μg/ml). These mutagen concentrations resulted in a 99% reduction of progeny virus. Mutants, selected for an inability to produce multinucleated syncytia at 39.5°, were plaque purified an additional time.

Animal Inoculations. Four-week-old BALB/c St mice were inoculated intracerebrally with 0.05 ml of virus. In other experiments, mice were infected by intranasal instillation of virus.

Histology and Electron Microscopy. After fixation with Bouin's solution, tissues were embedded in paraffin, sectioned, and stained with hematoxylin–eosin. Alternatively, mice were perfused via the heart with 2.5% phosphate-buffered glutaraldehyde. The spinal cords were embedded in araldite and stained with *para*-phenylenediamine. For electron microscopy, sections were stained with uranyl acetate and lead citrate and examined with a Siemens 101 electron microscope.

RESULTS AND DISCUSSION

Sixteen genetically stable ts mutants were isolated. Four representative mutants, ts7, ts8, ts11, and ts15, were selected for animal experiments *in vivo*. To control for the effects of cell passage upon the neurovirulence of MHV, we further plaque purified (twice) and passaged (three times) the parental virus in NCTC-1469 cells, so as to be equivalent to the isolation sequence of the mutants. This virus was then used as the wild-type virus.

Attenuation of Neurovirulence. Intracerebral inoculation with as few as 2 PFU of the wild-type virus killed 50% of 4-week-old BALB/c St mice, with the majority of deaths occurring between 4 and 6 days after inoculation (Table 1). In contrast, the ts mutants were highly attenuated since intracranial inoculation of 10,000 PFU of any of these mutants produced fewer than 50% mortalities. The attenuation is not due to an interference phenomenon because inoculation of 1000 PFU of ts8 did not result in any mortalities while 500 PFU of the wild-type virus produced death in all animals. The incidence of acute fatal encephalomyelitis after inoculation of the various ts mutants ranged from 3 to 40%. Attenuation was also manifested by an increase in survival time. Typically, mice did not die until 8–11 days after infection. Attenuation appeared linked to the ts mutation since selection for a revertant (ts^+ 11) resulted in the restoration of full virulence (LD_{50} = 1.3 PFU, as determined by injection of multiple virus doses and analyzed by the Reed–Muench method). In contrast, ts11 recloned at the permissive temperature ($ts11_{cl}$) was not altered in attenuation (Table 1). Animals that succumbed to the fatal necrotizing encephalomyelitis had severe lesions in both gray and white matter. These lesions are distinguished from those seen in the nonfatal disease which are restricted to the white matter and consist of demyelination rather than necrosis.

Induction of Demyelination. To avoid misinterpreting artifacts due to needle trauma to the brain, we limited the histological criterion of demyelination to lesions in the spinal

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Abbreviations: MHV, mouse hepatitis virus; ts, temperature sensitive; PFU, plaque-forming units.

† To whom reprint requests should be addressed.

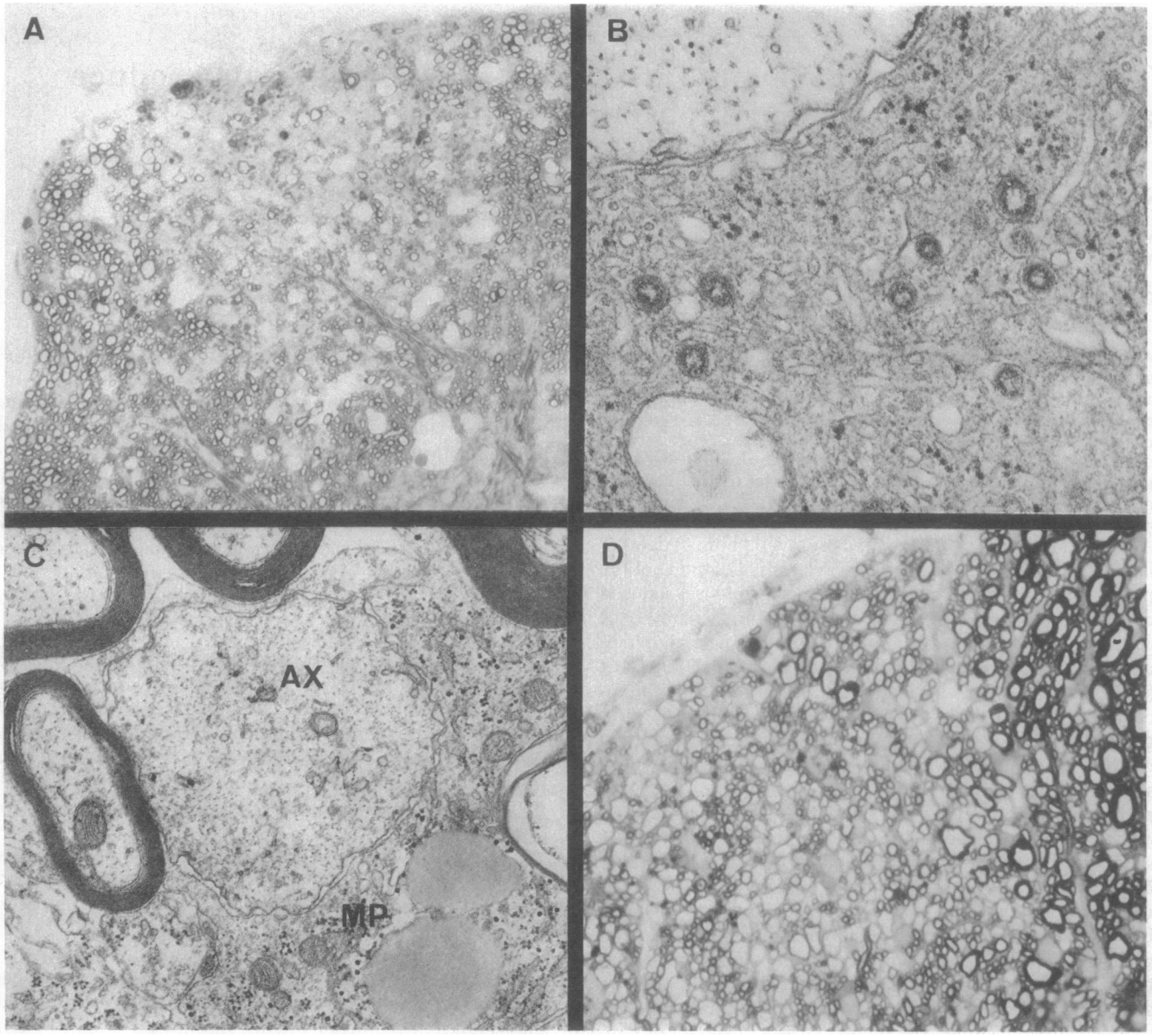

FIG. 1. Demyelination in the spinal cord of BALB/c St mice induced by ts mutant 8 of MHV (JHM strain). (*A*) Patchy demyelinated lesion in the white matter of a mouse 14 days after infection, stained with *para*-phenylenediamine. (×250.) (*B*) Oligodendroglia containing virus particles within the endoplasmic reticulum 5 days after infection. (×70,000.) (*C*) Large demyelinated axon (AX) surrounded by a macrophage (MP) 5 days after infection. (×20,000.) (*D*) Demyelinated lesion 54 days after infection. Remyelination associated with oligodendroglial proliferation was detected in this lesion. (×800.)

cord. The ability of the wild-type virus to produce demyelination was, as previously reported (5), highly dependent upon virus dose. The highest incidence of demyelination followed administration of a virus dose (10 PFU) that also resulted in 75% mortalities. When the infectious dose was decreased so that more animals survived, the incidence of demyelination among the survivors concomitantly decreased. Thus it is apparent that the experimental system provided by the wild-type virus does not lend itself to a detailed study of the biology and the molecular pathology of MHV-induced demyelination.

In contrast to the results obtained with wild-type virus, we noted that the use of ts mutants provided an excellent model for study of demyelination. Demyelination was observed in over half of the mice infected with ts8, while only 1 of 38 mice developed the acute fatal disease. Similarly, demyelination occurred in 75% of mice inoculated with ts11 and 22% of mice injected with ts7 (Table 1). Of interest was ts15, which produced demyelination in only 5% (1/21) of the mice despite replication of the virus *in vivo* (data not shown).

In these experiments, paraffin-embedded tissues were examined as a routine laboratory assay for evidence of demyelination. In other experiments with ts8 (10,000 PFU), 32 mice selected at random were perfused and their spinal cords were embedded in resin and stained with *para*-phenylenediamine. This method preserves axons and is a more sensitive index of demyelination. Demyelination was observed in all 32 mice infected with ts8 (10,000 PFU), while less than 2% of the mice developed the acute fatal disease (Table 1). Thus, with ts8 it will now be possible to examine in detail the mechanisms leading to demyelination in the absence of the acute fatal disease.

Pathology of Demyelination. The lesions induced in the spinal cord by the ts mutants consisted of well-defined areas of demyelination (Fig. 1*A*). Viral antigens were seen 2–4 days after infection in oligodendrocytes by using a monospecific

Table 1. Neurovirulence and induction of demyelination by MHV

Virus	Infectious dose*	Survival†	Demyelination‡
wt	10	3/12	2/3
wt	2	6/12	2/6
wt	0.4	9/11	1/9
ts7	10,000	9/12	2/9
ts8	10,000	114/115	12/12§
ts8	10,000	37/38	7/12
ts8	1,000	12/12	6/12
ts11	10,000	16/23	12/16
ts15	10,000	21/25	1/21
ts^+11¶	3	4/10	0/4
$ts11_{cl}$‖	10,000	6/10	5/6

wt, wild type.
* PFU/ml.
† Eleven days after inoculation, number positive per total number studied.
‡ Demyelination noted in the surviving mice; number positive per total number studied. Histopathologic study done on routine paraffin-embedded tissues.
§ Twelve mice were selected at random. Histopathologic study done on tissues embedded in resin and stained with *para*-phenylenediamine. Similar results were obtained with 20 mice from two other experiments.
¶ Ts^+ revertant virus isolated from a wild-type plaque at 39.5°.
‖ Ts mutant virus recloned at 34°.

fluoresceinated antibody to MHV. Similarly, virus particles were detected in oligodendroglial cells early after infection (Fig. 1*B*). The spherical virions were observed budding into the cisterns of endoplasmic reticulum. Macrophages were also observed removing the disintegrating myelin sheaths, leaving bare axons (Fig. 1*C*). Large areas of demyelination were noted in more advanced stages (Fig. 1*D*). Remyelination associated with oligodendroglial proliferation, as described by Herndon *et al.* (11) in mice recovering from infection with the wild-type virus, was also noted in lesions more than 1 month after infection.

Demyelination after Intranasal Inoculation. It was important to determine whether demyelination could occur after a natural route of injection. When eight mice were inoculated intranasally with 10,000 PFU of ts8, lesions were observed in the spinal cords of four mice. Of these mice, two exhibited extensive demyelination.

Mutants Are Associated with Different Frequencies of Demyelination. The ts mutants differed in their abilities to produce demyelination. These differences, as discussed below, cannot be readily attributed to factors such as lethality of the virus *in vivo*, partial activity ("leakiness") of the mutants *in vitro*, reversion frequency, or ability to replicate at the body temperature of a mouse (37°).

The incidence of demyelination induced by the ts mutants was not related to the percentage of fatalities. ts8 caused few fatalities, yet produced demyelination. Furthermore, ts8 was still able to produce demyelination at a lower infectious dose (1000 PFU) (Table 1). In addition, ts7 and ts11 caused equal numbers of fatalities, yet differed in the incidence of demyelination.

The differences in frequency of demyelination cannot be attributed to the partial activity or "leakiness" of the mutants *in vitro* at the nonpermissive temperature. Both ts8 and ts15 exhibit some replication at 39.5° (Table 2). The progeny virus produced under these conditions were still temperature sensitive. Thus, these viruses, although similarly "leaky" (i.e., produced similar infectious titers at the nonpermissive temperature), differed dramatically in ability to produce demyelination. Furthermore, ts11, which does not exhibit any detectable replication at 39.5°, produces demyelination with high frequency. In addition, all of the mutants are of similar genetic stability (reversion frequencies $10^{-3.4}$ to $10^{-4.9}$) (Table 2) and viruses isolated from mutant-infected animals were found to still be temperature sensitive (data not shown). Finally, all of the mutants replicated efficiently *in vitro* at 37°.

CONCLUSIONS

Selection for ts mutants resulted in an enhancement of the ability of MHV type 4 to produce demyelination. With this reliable model, it is now possible to study in depth the pathogenic mechanism of virus-induced demyelination. In addition, the availability of ts mutants that produce demyelination with either extremely low or high frequency suggests a theoretical possibility of determining which gene product(s) are required for demyelination.

This is publication no. 1419 from the Department of Immunopathology, Scripps Clinic and Research Foundation, La Jolla, CA 92037. We thank Peggy Farness for excellent technical assistance and Sally Hendrix and Phyllis Minick for preparation of the manuscript. This research was supported by U.S. Public Health Service Grants AI-14290, NS-09053, NS-12428, and NS-14068, and Biomedical Research Support Program Grant RR0-5514.

Table 2. Preliminary physiological characterization of representative ts mutants

Property	Virus: wt	ts7	ts8	ts11	ts15
EOP:* 39.5/34°	1.3×10^0	1.3×10^{-5}	4.0×10^{-4}	1.6×10^{-5}	4.5×10^{-4}
Virus titers:† 34°	1.8×10^5	1.4×10^4	1.1×10^4	4.3×10^3	1.4×10^4
37°	5.5×10^5	2.4×10^4	1.1×10^5	4.8×10^5	9.3×10^4
39.5°	3.6×10^5	$<2.5 \times 10^0$	3.1×10^2	$<2.5 \times 10^0$	5.5×10^2
"Leakiness" ‡		−	+	−	+
Cytoplasmic antigen synthesis:§ 34°	+	+	+	+	+
39.5°	+	−	+	−	+
Surface antigen synthesis:¶ 34°	+	+	+	+	+
39.5°	+	−	+	−	+

* Efficiency of plating on NCTC-1469 cells. Wild-type (wt) (revertant) plaques were counted at 39.5°.
† PFU/ml 12 hr after infection [inoculum 4×10^4 PFU (multiplicity of infection $\simeq 0.1$)] of NCTC-1469 cells.
‡ Some functional activity at 39.5° resulting in mutant progeny virus.
§ Indirect immunofluorescence of acetone-fixed cells 10 hr (34°) or 6 hr (39.5°) after infection.
¶ Indirect immunofluorescence of living cells 10 hr after infection.

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