

Review

## Theiler's Murine Encephalomyelitis Virus (TMEV): the Role of a Small Out-of-Frame Protein in Viral Persistence and Demyelination

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**SUMMARY:** Theiler's murine encephalomyelitis virus (TMEV) belongs to the genus *Cardiovirus* of the family *Picornaviridae* and is divided into two subgroups on the basis of different biological activities. GDVII subgroup strains produce acute and fatal polioencephalomyelitis in mice with no virus persistence. In contrast, DA or TO subgroup strains cause an early nonfatal polioencephalomyelitis. TMEV is thought to be an excellent animal model for the human demyelinating disease, multiple sclerosis. Data suggest that macrophages are a major reservoir harboring the virus. A small out-of-frame protein designated L\* is synthesized in DA subgroup strains from an alternative, out-of-frame, initiation site. Studies of a DA mutant virus, having an ACG rather than an AUG and therefore does not synthesize L\* protein, demonstrate that this protein is important for virus growth in particular cell types and is critical for DA-induced demyelinating disease and virus persistence. In addition, TMEV can be used as a vector for delivering foreign sequences into the central nervous system.

### Introduction

Theiler's murine encephalomyelitis virus (TMEV) belongs to the genus *Cardiovirus* of the family *Picornaviridae*. Strains of TMEV are divided into two subgroups on the basis of different biological activities (1-3). GDVII subgroup strains produce acute and fatal polioencephalomyelitis with no virus persistence in mice (1-4). In contrast, DA or TO subgroup strains induce an early nonfatal polioencephalomyelitis of weanling mice followed by chronic demyelination. Virus persists in the demyelinated spinal cord in the face of a vigorous humoral immune response. Pathological features of demyelination with inflammatory cell infiltration are reminiscent of those found in the human demyelinating disease, multiple sclerosis (MS) (1-3). Therefore, TMEV-induced demyelinating disease is thought to be an excellent animal model for MS. In the TMEV animal model, however, the mechanisms of virus persistence and demyelination remain unclear. In this article, we will focus on a small alternatively initiated viral protein (5,6) that plays a key role in the late demyelinating disease. The protein is only synthesized in DA subgroup strains and is therefore thought to be a key determinant of the different biological activities observed between the two subgroup strains of TMEV.

### In vivo and in vitro biological activities of TMEV subgroups

Although TMEV was originally classified in the genus *Enterovirus*, it is now classified in the genus *Cardiovirus* based on sequence homology (7-9). All strains of TMEV are grouped together because they are of one serotype and can be neutralized by the same polyclonal antiserum (1). TMEV strains produce enteric and neurologic diseases in mice, and they can be divided into two subgroups on the basis of their different in vivo and

Table 1. Characteristic in vivo and in vitro activity of the TMEV subgroup

	Subgroup	
	DA(TO)	GDVII
Representative strains:	DA, TO, WW, BeAn <i>et al.</i>	GDVII, FA
in vivo activities:		
Neurovirulence	+	++
Acute polioencephalomyelitis	±	+
Chronic demyelination	+	-
Persistence	+	-
in vitro activities:		
Plaque size in BHK-21 cells (mm)	small (<0.3)	large (2.5)
Ultrastructure in BHK-21 cells	membranous structure	crystalline array
Growth in macrophages	+	-

in vitro biological activities (Table 1).

GDVII subgroup strains, typified by the GDVII strain, are highly virulent and cause an acute fatal polioencephalomyelitis in weanling mice after intracerebral and peripheral routes of inoculation (10). After an incubation period of usually less than 2 weeks, infected mice show circling, cachexia, and ruffled fur, in addition to flaccid paralysis. Almost all the infected mice die. Neither virus persistence nor demyelination is observed in the few surviving mice (4). Histological examination reveals a severe necrosis of neurons of the hippocampus, cortex, and spinal cord anterior horn, with microgliosis, neuronophagia, and inflammatory cell infiltration (1-3). Tsunoda *et al.* recently reported that the apoptosis of neurons may be responsible for the fatal outcome in GDVII infection (11).

In contrast to GDVII subgroup strains, DA subgroup strains cause a biphasic disease after intracerebral inoculation (1-3,12,13). The early disease, which appears 1-3 weeks after inoculation, has clinical and pathological features that are similar to those seen in GDVII subgroup strains, but milder.

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Perivascular inflammation and necrosis are observed in the gray matter, primarily in the spinal cord anterior horn, cerebral cortex, and the hippocampus. Mice recover from the early disease and then develop a chronic, progressive white matter-demyelinating disease beginning about 1-month postinoculation (p.i.). Clinical signs include spastic paralysis, inactivity, urinary incontinence, and priapism (1,3). The demyelination primarily affects the spinal cord, with the white matter of the brain being spared. The pathology is reminiscent of MS, with inflammatory cell infiltration and a loss of myelin occurring with a relative preservation of axons. Mononuclear and plasma cells infiltrate the leptomeninges, perivascular areas, and adjacent white matter (Fig. 1). Electron microscopic study demonstrated the stripping of myelin lamellae in response to an invasion of mononuclear cells similar to that seen in experimental allergic encephalitis (14). This chronic demyelinating disease in mice is thought to be an appropriate animal model for MS (1-3).

As shown in Table 1, TMEV also shows subgroup-specific *in vitro* activities. In BHK-21 cells, DA subgroup strains produce small plaques (less than 0.3 mm in diameter), whereas GDVII subgroup strains show a plaque size of 2.5 mm in diameter (4). Electron microscopy demonstrated another feature differentiating TMEV subgroups. During late infection

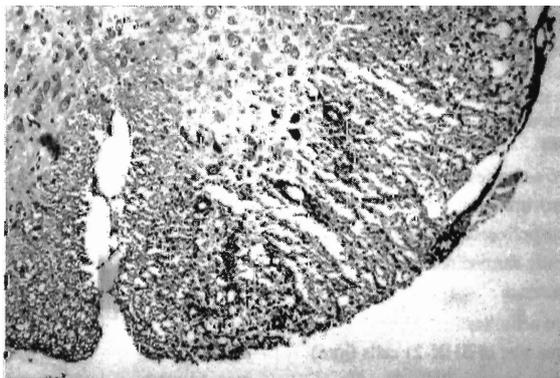


Fig. 1. TMEV-induced demyelination. Spinal cord from an SJL/J mouse approximately 8 weeks postinoculation with a DA strain of TMEV. Demyelination with inflammatory cell infiltration is found in the anterior funiculus of the spinal cord. Klüver-Barrera stain,  $\times 50$ .

(7 to 12 h p.i.) in BHK-21 cells, the GDVII strain forms well-developed crystalline arrays in the cytoplasm of infected cells and is readily released upon cell lysis (15). In contrast, the DA strain forms membranous structures consisting of two-membrane units enclosing a single layer of viral particles and does not appear to be released freely upon cell lysis (15). This observation is of interest because such an arrangement may prohibit or hinder the exposure of virus particles to the immune system. Subgroup-specific host cell-restricted infection is another *in vitro* feature distinguishing the two subgroups (16). In BHK-21 cells, both DA and GDVII strains productively grow, although the titers of the DA strain are about 1 log unit lower than those of the GDVII strain. On the other hand, the growth kinetics is remarkably different between the two strains in J774-1 cells, a macrophage-like cell line derived from a mouse tumor. The GDVII strain does not actively replicate in these cells, although there is a significant inhibition of cellular protein synthesis. In contrast, the DA strain productively infects these cells with little if any cellular protein synthesis shutoff (Fig. 2) (16). A subgroup-specific growth difference is also observed in other macrophage cell lines (Table 2) (17).

#### Target cell for virus persistence

As described above, both subgroup strains of TMEV primarily infect neurons during acute polioencephalomyelitis (1-3). It is interesting that DA viral antigen and RNA that are present in neurons in the acute stage are not observed in

Table 2. TMEV growth in neuronal and non-neuronal cell lines

Cell line	Cell type	Virus growth		
		DA	GDVII	DAL*-1
C-1300	Neuroblastoma	+	+	+
OS3	Oligodendrocyte/ type-2 astrocyte	+	+	+
C6	Glioma	+	+	+
G26-20	Glioma	+	+	+
J774-1	Macrophage	+	-	-
RAW264.7	Macrophage	+	-	-
P338D <sub>1</sub>	Macrophage	+	-	-
L-929	Fibroblast	+	+	+

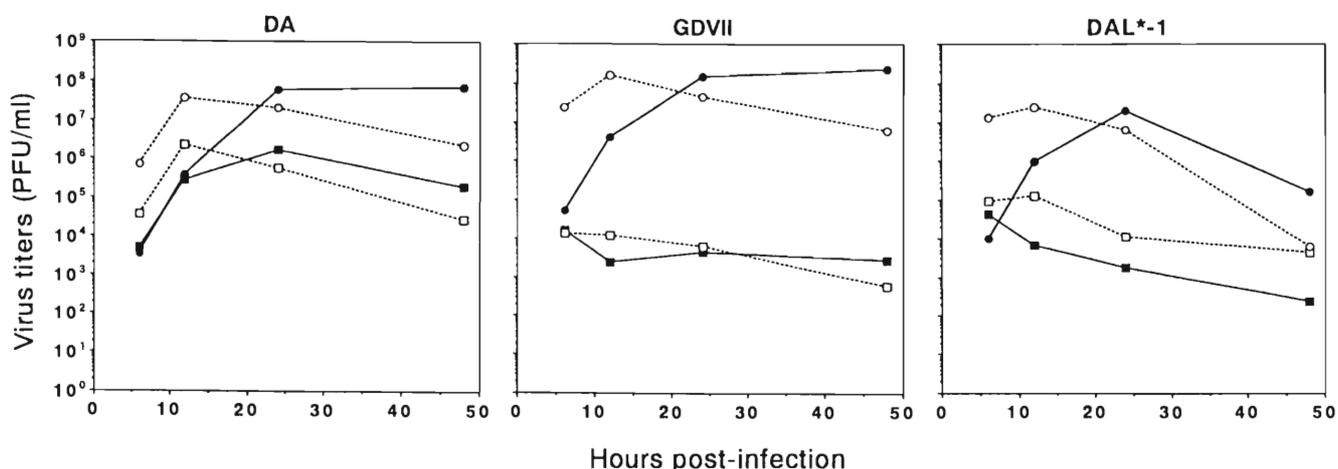


Fig. 2. Kinetics of DA, GDVII, and DAL\*-1 virus growth in BHK-21 and J774-1 cells. The culture supernatants (solid line, closed symbol) and cell lysates (broken line, open symbol) of infected BHK-21 (circle) or J774-1 (square) cells were harvested at the indicated times and subjected to an infectivity assay by a standard plaque titration on BHK-21 cells. Data are expressed as the mean titer from three independent experiments.

neurons during the chronic stage (18,19). The actual localization of DA subgroup strains in the chronic demyelinating stage is somewhat controversial. Rodriguez et al. (18) used immunoelectron microscopy to detect the presence of viral antigen in neurons and astrocytes in the acute stage of infection as well as in macrophages, astrocytes, and oligodendrocytes at 28 days p.i., and reported that most infected cells at 45 days p.i. and later were oligodendrocytes. An in situ hybridization study demonstrated that a small amount of viral RNA was present within glial cells in the white matter of the spinal cord during the chronic demyelinating stage. It was, however, impossible to identify the labeled glial cells as oligodendrocytes or astrocytes on purely morphological criteria (19). Ohara et al. reported that a primary culture of oligodendrocytes can be infected with both subgroup strains (20). Using a simultaneous immunoperoxidase-in situ hybridization assay, Aubert et al. (21) quantitatively analyzed the population of DA-infected cells in the chronic stage, and reported that 25-40% were oligodendrocytes, 5-10% astrocytes, 10% microglia/macrophages, and the rest unknown.

In contrast, other investigators claimed that the virus resided in macrophages in the chronic demyelinated stage. Using ultrastructural immunohistochemical techniques, researchers observed viral inclusions in macrophages in and around demyelinating lesions (22). It was also reported that infectious BeAn virus, which is a demyelinating TMEV strain similar to DA, was recovered from infiltrating mononuclear cells isolated directly from the central nervous system (CNS) of chronically infected mice (23). Two-color immunofluorescent staining with conventional and confocal microscopy showed that the viral antigen burden was predominantly within macrophages infiltrating demyelinating lesions (24). A small fraction of cultured primary brain macrophages could be efficiently infected with the DA strain without significant cytopathic effects (25). Rossi et al. (26) also examined the types of cells infected at different times p.i. Approximately 10% of infected cells were astrocytes at any time p.i. The number of infected macrophages increased with time and reached a plateau by 21 days p.i. when approximately 50% of infected cells were macrophages. In addition, the depletion of blood-borne macrophages by dichloromethylene diphosphonate prevented virus persistence in mice infected with the DA strain, further emphasizing the importance of macrophages in the late demyelinating disease (26).

From the above data, it appears likely that macrophages

are the major cell containing the persistent viral genome, although the virus can also be found in astrocytes and oligodendrocytes.

#### A small out-of-frame protein synthesized only in DA subgroup strains

TMEV belongs to the family *Picornaviridae* and infects the gastrointestinal tract and the CNS (1-3). The virion has an icosahedron shape about 28 nm in diameter without a lipid envelope. A single-stranded RNA is enclosed by the shell consisting of four capsid proteins, referred to as VP1, VP2, VP3, and VP4. The three-dimensional structure was resolved by means of X-ray crystallography in the early 1990s (27,28). The nucleotide and predicted amino acid sequences were determined in late 1980s (7, 8, 29); neutralizing epitopes have been identified (30-32), and full-length infectious cDNAs of TMEV strains were constructed (33,34).

The genome is of positive sense and is approximately 8100 nucleotides long. A long open reading frame (ORF) between the 5' and 3' noncoding regions is translated into a long polyprotein, which is then cleaved into L, P1, P2, and P3. L is a small leader protein present only in cardioviruses and aphthoviruses. Although the function of L protein is not known, L plays a role in host cell-restricted infection (35).

A noteworthy feature regarding the L region is that DA subgroup strains have an alternative translation initiation codon within the L coding region at nucleotide (nt) 1079, in addition to the authentic initiation site for the polyprotein at the amino terminus of L (nt 1066) (5, 6) (Fig. 3). This alternative initiation site is out-of-frame with the polyprotein and is used to translate a small 17 kDa protein, designated L\*. The synthesis of L\* protein is subgroup-specific because the alternative initiation site is present in DA, TO, WW, BeAn, and Yale strains, but not the GDVII or FA strains (where the L\* AUG is substituted by an ACG) (36). Therefore, this unique protein was thought to potentially play a role as a determinant of the different activities observed in both virus subgroups.

#### Role of L\* protein in TMEV growth in macrophages

As previously noted, DA grows well in J774-1 cells, while GDVII does not. The importance of L\* protein in this difference was demonstrated in studies with a mutant virus, DAL\*-1, which has an ACG rather than an AUG and therefore does not synthesize L\* protein (Fig. 3). Takata et al. found

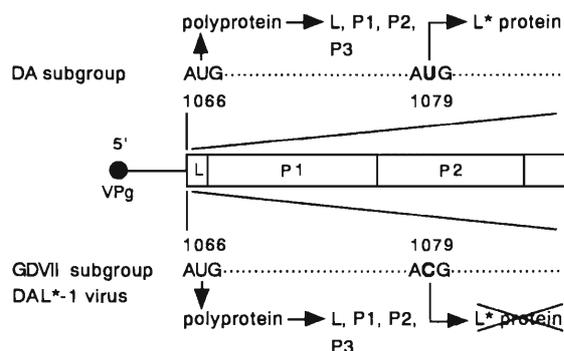


Fig. 3. Authentic and alternative initiation sites in TMEV. An authentic initiation site is located at nucleotide (nt) 1066 in both strains and a mutant virus, DAL\*-1. From this AUG, a long polyprotein is translated and subsequently cleaved into L protein, P1, P2, and P3. On the other hand, an alternative, out-of-frame, initiation site that is only present in DA subgroup strains is located at nt 1079 and is used to translate a small 17 kDa protein, L\*. GDVII subgroup strains do not synthesize L\* since U at nt 1080 is substituted with C. The DAL\*-1 virus also fails to synthesize L\* because the U is substituted with C (as a result of site-directed mutagenesis).

that the DAL\*-1 virus failed to grow in J774-1 macrophage cells, whereas the virus grew well in BHK-21 cells, suggesting that L\* protein plays a role in the TMEV subgroup-specific host cell-restricted infection (Fig. 2). The restriction of virus growth in these cells is regulated at the step of viral RNA synthesis (37). Obuchi et al. found that the DAL\*-1 and GDVII viruses, neither of which produce L\* protein, failed to grow in macrophage cell lines, but grew as well as DA in other neural and non-neural cell lines (17) (Table 2). In addition, a host cell-specific antiapoptotic effect was recently demonstrated in a macrophage cell line, P388D1 (38). These features are of interest because macrophages are one of the major cell types for TMEV persistence during the stage of chronic demyelination, and because virus growth and minimum host cell damage are critical for maintenance of the viral genome and viral persistence (39).

### Role of L\* protein in virus persistence and demyelination

There are a number of reports demonstrating that L\* protein plays a role in virus persistence and demyelination. The DAL\*-1 virus produces an early acute polioencephalomyelitis similar to parental DA. However, the viral RNA genome was no longer detected in the spinal cord of mice 6 weeks p.i. (38). In addition, there was minimal if any evidence of demyelination or inflammation in the spinal cord at this time (40). It was also demonstrated that L\* protein appears to inhibit the generation of H-2K-restricted TMEV-specific cytotoxicity, therefore permitting a persistent infection in susceptible mouse strains (41). Yamasaki et al. recently reported a variation in the utilization of translation initiation at the polyprotein's AUG versus the L\* AUG (42). These investigators proposed that L\* (rather than the polyprotein) is preferentially synthesized in certain CNS cells (e.g., microglial cells) following infection with DA subgroup strains, leading to a restricted expression of the virus. The production of only small amounts of capsid protein would decrease neurovirulence in the acute stage and also foster a persistence of the virus in the chronic stage.

The above data indicate that L\* protein is a key determinant of TMEV persistence, with subsequent inflammatory demyelination in the CNS similar to that in MS. All the data have been obtained from studies using the mutant virus, DAL\*-1, which does not synthesize L\* protein. Additional data using a recombinant virus which synthesizes L\* protein in the background of the parental GDVII virus would be valuable in order to confirm the role of L\* protein.

### TMEV as a vector for delivery of foreign genes into the CNS

The aphthoviruses and cardioviruses of *Picornaviridae* contain an L coding region at the start of the ORF (9). In the case of foot-and-mouth disease virus, an aphthovirus, L protein has been shown to have two proteolytic functions (3), autocatalytic cleavage from the viral polyprotein and cleavage of the cap-binding protein complex, p220. In contrast, the function of L protein of the cardiovirus is unknown. The L coding region of TMEV, a cardiovirus, is at the 5' end of the polyprotein's ORF starting from the AUG at nt 1066. Interestingly, Kong et al. (35) reported that other sequences unrelated to TMEV can be inserted into the L coding region with no loss of infectivity in some cells. Obuchi et al. (43) inserted

the lymphotoxin (LT) gene into the L region, resulting in an expression of LT, which was demonstrated by cytotoxicity against L-929 cells (one of the biological activities of LT).

The above studies indicate that TMEV may be used as a vector for delivering foreign sequences into the CNS (43, 44). One potential shortcoming of this approach is that the coding sequence of L protein overlaps with that of L\* protein, as a result, insertion of a foreign sequence into the L coding region interrupts L\* translation. Since L\* protein is important for virus persistence, interruption of L\* translation prevents virus persistence and therefore prevents continuous expression of the transgene in the CNS. Therefore, a more appropriate site of insertion must be explored.

Another potential problem related to the use of the DA strain as a virus vector is the probability that the virus will induce demyelination. Of interest is the recent report by Sato et al. (32) concerning a DA mutant virus in which lysine changes to asparagine at amino acid 141 in VP2; this mutation persists but does not cause demyelination. There is a continuing role for genetic manipulation of DA virus in the future in order to produce non-pathogenic vectors.

### Conclusion

DA subgroup strains of TMEV cause demyelination in mice that is reminiscent of that seen in the human demyelinating disease, MS. Although the mechanism(s) of demyelination remains to be clarified, a small out-of-frame protein designated L\* seems to play an important role in virus persistence and disease pathogenesis. L\* protein is synthesized in DA subgroup strains from an alternative, out-of-frame, initiation site. Studies of a DA mutant virus which has an ACG rather than an AUG and therefore does not synthesize L\* protein demonstrated that this protein is important for virus growth in particular cell types and is critical for DA-induced demyelinating disease and virus persistence. Recent studies have shown that TMEV can be used as a vector for delivering foreign sequences into the CNS. The availability of a murine experimental host susceptible to DA infection provides an attractive model system for an elucidation of the pathogenesis of virus-induced demyelination and/or the development of gene therapies for intractable human neurological diseases.

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