Development of Postinfection Epilepsy After Theiler’s Virus Infection of C57BL/6 Mice

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Abstract
Viral infection of the central nervous system can lead to long-term neurologic defects, including increased risk for the development of epilepsy. We describe the development of the first mouse model of viral-induced epilepsy after intracerebral infection with Theiler’s murine encephalomyelitis virus. Mice were monitored with long-term video-electroencephalogram at multiple time points after infection. Most mice exhibited short-term symptomatic seizures within 3 to 7 days of infection. This was followed by a distinct latent period in which no seizures were observed. Prolonged video-electroencephalogram recordings at 2, 4, and 7 months after the initial infection revealed that a significant proportion of the mice developed profound, spontaneous epileptic seizures. Neuropathologic examination revealed hippocampal sclerosis in animals with epilepsy. Theiler’s murine encephalomyelitis virus–infected C57BL/6 mice represent a novel "hit-and-run" model to investigate mechanisms underlying viral-induced short-term symptomatic seizures, epileptogenesis, and epilepsy. Importantly, this model will also be useful to investigate novel therapies for the treatment and prevention of epilepsy.

Key Words: Animal model, Epilepsy, Epileptogenesis, Hippocampus, Inflammation, Seizure, Virus.

INTRODUCTION
Epilepsy is one of the most prevalent neurologic disorders, affecting approximately 2.5 million people in the United States and more than 50 million people worldwide (1). Although a significant proportion of seizure disorders have no definable cause (idiopathic), it is estimated that up to 50% of epilepsy cases are associated with previous neurologic injury (acquired epilepsy) (2). Central nervous system (CNS) infection is an example of brain injury that can lead to the development of acquired epilepsy. Particularly, for patients with viral encephalitis, the risk of epilepsy is increased 16-fold, and the risk persists for at least 15 years after infection (3). Furthermore, epilepsy after CNS infection is often intractable to available pharmacological and surgical interventions (4).

Despite its significance and overall prevalence, our understanding of postinfection epilepsy is limited; this is partly due to a paucity of animal models that accurately reproduce the pathophysiology of the human disorder. Studies using currently available animal models have proved useful in documenting neuropathologic, electrophysiologic, and immunologic alterations during the short-term infection (5–7). One of the primary limitations of the current models, however, is a high incidence of mortality during the short-term infection. For example, intranasal inoculation of West Nile virus causes symptomatic limbic seizures in mice, but the virus induces short-term fatal encephalitis thereby precluding any long-term study of the mechanisms underlying the epileptogenesis (5). Similarly, intracerebral injection of measles virus in mice leads to short-term encephalitis, behavioral seizures, and death approximately 7 days postinjection (PI) (6). Corneal inoculation with herpes simplex virus type 1 induces short-term seizures as well as long-term hippocampal excitability and increased seizure susceptibility in mice that survive the short-term infection (7). Although this model provides an excellent experimental system to study herpes simplex virus type 1 infection, its applicability to other infection-seizure pathologies is limited because the virus persists throughout the life of the animal. To date, there have been no reports of an animal model that displays overt long-term defects such as spontaneous recurrent seizures, the defining feature of epilepsy. Thus, new experimental models in which animals clear the virus, recover from the initial infection, and subsequently develop spontaneous epileptic seizures are needed.

Recently, we reported that C57BL/6 (B6) mice infected with the Daniel’s strain of Theiler’s murine encephalomyelitis virus (Theiler’s virus or TMEV) develop short-term encephalitic seizures (8, 9). Theiler’s murine encephalomyelitis virus belongs to the Picornaviridae family, a relatively large family of nonenveloped, positive-stranded RNA viruses (10). Intracerebral inoculation of certain mouse strains (e.g. SJL/J) results in a biphasic disease consisting of short-term encephalitis and long-term demyelination (11). In contrast, other mouse strains such as B6 develop only short-term encephalitis and completely clear the virus within 2 to 4 weeks PI (12). In addition to short-term symptomatic seizures, B6 mice also display significant pyramidal neuron cell death and increased transforming growth factor β (TGF-β) in the hippocampus during
the short-term encephalitic period (8, 9). Furthermore, TMEV infection accompanied by short-term symptomatic seizures results in chronically reduced seizure thresholds and increased susceptibility to kindling (9). These findings suggest that TMEV infection leads to an increased propensity for the development of an epileptogenic circuit in B6 mice.

Here, we used video-electroencephalogram (VEEG) monitoring to characterize the natural progression of short-term encephalitic seizures in TMEV-infected B6 mice and to determine whether these mice subsequently develop epilepsy. In addition, our histologic data confirm previous studies demonstrating pathologic features suggestive of hippocampal sclerosis in epileptic mice, including neuronal cell death, reactive astrogliosis, and enlargement of the lateral ventricles due to hippocampal degeneration. This report provides comprehensive characterization of the short-term symptomatic seizures after viral inoculation and details the progression of the development of epilepsy after an undefined latent period. Together, the data establish TMEV infection of B6 mice as a novel model of postinfection epilepsy.

**MATERIALS AND METHODS**

**Animals**

Male B6 mice (Jackson Laboratories, Bar Harbor, ME), 4 to 5 weeks old, were used for all experiments. Animals were kept on a 12-hour light-dark cycle and allowed free access to food and water. All animal care and experimental manipulations were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Utah Institutional Animal Care and Use Committee.

**Infection of Mice**

The DA strain of TMEV is a tissue culture-attenuated virus maintained in baby hamster kidney-21 cells (13). Under isoflurane anesthesia, mice were injected intracerebrally with $2 \times 10^4$ plaque-forming units of TMEV in a total volume of 20 μL, as previously described (9). Control mice were injected with sterile phosphate-buffered saline (PBS). Injections were made in the right cerebral hemisphere halfway between the back of the eye and the ear, at a 45-degree angle from the top of the skull. A sterile 28-gauge 0.5-mL insulin syringe was fitted with a William’s collar that allowed an injection depth of 2.5 mm was used for injections.

**EEG Electrode Implantation**

Mice were anesthetized with ketamine hydrochloride (120 mg/kg) and xylazine (12 mg/kg) intraperitoneally and injected with penicillin (15,000 U) and dexamethasone (1.5 mg/kg) subcutaneously to prevent postsurgical infections. The skull was exposed, and 3 holes on each side of the midline were drilled. Using a 3-channel stainless steel electrode unit (MS333/1-A; Plastics One, Roanoke, VA), 2 electrodes were placed bilaterally over the frontoparietal cortex and 1 ground electrode was placed over the caudal cortical region. Stainless steel mounting screws (80-96 X 1/16; Plastics One) were placed in the remaining 3 holes in the skull, and the entire assembly was anchored with dental cement. Mice were administered an analgesic (buprenorphine; 0.1 mg/kg) and 1 mL of lactated Ringer’s solution (both subcutaneously) and allowed to recover from the anesthesia under a heat lamp for 1 hour. All mice were individually housed in Plexiglas cages and allowed to recover for 1 week after surgery.

**Short-Term VEEG Monitoring**

For short-term VEEG studies, electrodes were implanted in 4-week-old mice. At 7 days after implantation, the mice were placed under 24-hour VEEG monitoring in custom Plexiglas cages that allowed free access to food and water. Recordings were performed via long flexible cables (Plastics One) in freely moving, nonanesthetized mice. After baseline recordings, mice were randomly injected intracisternally with either TMEV or PBS and immediately returned to their labeled recording cages. Sixteen TMEV-injected and 8 PBS-injected mice were monitored continuously for 2 weeks (Fig. 1A).

Electroencephalogram recordings were obtained at a frequency band of 1 to 50 Hz, amplified 5000×, and sampled at 500 Hz/channel (8 channels) using a Biopac digitizer and Biopac Acknowledge Software (Santa Barbara, CA). Video recordings of behavior were captured with EZ-Watch Pro infrared digital color cameras (Automated Video Systems, Salt Lake City, UT). An individual blinded to study groups analyzed video and EEG results for each animal. Electrographic seizures were defined as rhythmic spikes or sharp-wave discharges with amplitudes at least 2 times higher than baseline and lasting longer than 6 seconds. All electrographic activity was correlated with concurrent videotaped behavior. To be considered a seizure, electrographic activity had to occur with a Racine behavioral seizure correlate (see scoring criteria below). Abnormal electrographic activity that had large amplitude multispike morphology and either was associated with sudden cessation of activity or no distinct behavioral correlate was also noted and classified as epileptiform activity.

**Long-Term VEEG Monitoring**

Mice were injected with either TMEV (n = 30) or PBS (n = 9) at 5 weeks of age as described above and visually observed for seizures 2 h/d for 2 weeks. Seizure activity was scored according to the Racine 5-stage seizure scale (stage 1, chewing and drooling; stage 2, head nodding; stage 3, unilateral forelimb clonus; stage 4, rearing with bilateral forelimb clonus; and stage 5, rearing and falling [loss of postural control] progressing to generalized clonus) (14). Infected mice that displayed 1 or more seizures during this observation period (n = 23) and PBS-injected controls (n = 9) were used for long-term VEEG studies. At 7 weeks PI, electrodes were implanted, and VEEG monitoring commenced at 8 weeks PI.

**Immunohistochemistry**

Mice were anesthetized with pentobarbital (60 mg/kg, intraperitoneally) and perfused transcardially with 0.1 mol/L PBS followed by 4% paraformaldehyde (PFA; Sigma-Aldrich, St Louis, MO). Brains were then removed and postfixed overnight at 4°C in 4% PFA solution and cut coronally into 40-μm-thick sections with a vibratome. Slices were mounted onto glass slides and incubated for 1 hour with 4.5% normal goat serum.
Vector Labs, Burlingame, CA) and 0.3% Triton X (Sigma-Aldrich) in PBS. Excess solution was drained off, and slices were incubated overnight with primary antibodies for astrocytes (mouse anti-glial fibrillary acidic protein [GFAP] Alexa Fluor 488-conjugated, 1:1000 [MAB3402X; Millipore, Billerica, MA]) or neurons (mouse anti-NeuN, 1:1000 [MAB377; Millipore]) diluted in PBT (0.3% Triton X in PBS) and 4.5% normal goat serum. Sections were rinsed 3 times with PBS and then processed. For GFAP, sections were soaked for 30 minutes in DAPI (4',6-Diamidino-2-phenylindole), rinsed with ddH2O, and coverslipped with Vectashield mounting medium (Vector Labs). For NeuN, sections were incubated in biotinylated secondary antibody (goat antimouse, 1:2000; Vector Labs) followed by avidin-biotin-peroxidase complex (ABC Elite Kit; Vector Labs) according to the manufacturer’s specifications. The chromogen used for color development was 3,3’-diamino-benzidine (DAB Kit; Vector Labs). Sections were then dehydrated in 75%, 85%, and 95% ethyl alcohol (1 minute each), cleared in methyl salicylate (2 minutes), and coverslipped with Permount (Fisher Scientific, Waltham, MA).

**Timm/Cresyl Violet Staining**

Timm stain was used to visualize mossy fiber reorganization in the dentate gyrus, and cresyl violet counterstain was used to visualize cellular cytoarchitecture, as described by Hellier et al (15). Briefly, mice were perfused transcardially with an isotonic solution of 0.3% Triton X in PBS and 0.3% formaldehyde (Sigma-Aldrich) and 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA). Following perfusion, brains were removed and postfixed in 4% paraformaldehyde for 1–2 hours. Brains were then cryoprotected in sucrose (4°C) for 24–48 hours, cut into 30-μm-thick sections using a freezing microtome, and then stored in 0.01 M phosphate-buffered saline (PBS) until staining.

**FIGURE 1.** Chronology of procedures for short-term studies and representative EEG events during short-term Theiler’s murine encephalomyelitis virus (TMEV) infection. (A) Timeline of procedures. The top trace shows a normal PBS-injected control mouse baseline EEG. The bottom trace shows the events preceding and after a large-amplitude, high-frequency synchronous EEG event that corresponded to a Racine stage 5 behavioral seizure (lasting 1 minute) in a TMEV mouse. The numbered boxes (nos. 1–6) in these 2 traces are shown below in an expanded time scale. Baseline activity in TMEV mice was generally similar to that of control mice. The waveforms of the preictal and postictal spiking activity were usually similar, although the preictal spiking phase was typically much longer. Postictal suppression usually corresponded to behavioral arrest in the animal, followed by either intense grooming or wild running during the postictal spiking phase.
with 0.37% sodium sulfide (Sigma-Aldrich) then with 4% PFA. Brains were removed and postfixed overnight at 4°C in 4% PFA, then sectioned coronally (40 μm) with a vibratome. Alternate sections (5–8 sections total per mouse) were mounted, and slides were processed with either cresyl violet stain or modified Timm stain protocol followed by cresyl violet counterstaining, as described (16).

Cresyl violet–stained sections were used to analyze ventricular and hippocampal areas. To ensure that valid comparisons were made between TMEV- and PBS-injected mice, sections from similar anterior-posterior levels were used. The first section was taken immediately posterior to the olfactory bulb, and all sections were taken (excluding the cerebellum) thereafter. Every fifth section was stained with cresyl violet, and the ventricular and hippocampal areas were analyzed (4–8 sections per brain). Mossy fiber reorganization was assessed by analyzing the amount of dark reaction product in the granule and molecular cell layers of the dentate gyrus in Timm-stained sections. Staining was assessed semiquantitatively as follows: 0, no or only slight reaction product; 1, scattered reaction product; 2, patches of heavy reaction product or continuous band of intermediate staining; 3, dense continuous band of product (15). A score was given to each section from an animal and then the mean score was assigned to each.

Statistical Analysis

For parametric values, the mean ± SEM was calculated. One-way ANOVA with Tukey post tests was used to compare seizure frequency and duration. Data from these calculations are shown as mean ± SD. Unpaired 2-tailed Student t test was used to compare means of hippocampal and ventricular area in TMEV and PBS mice. GraphPad Prism (GraphPad Software, Inc., La Jolla, CA) was used for all statistical comparisons; p < 0.05 was considered significant.

RESULTS

Short-Term Encephalitic Seizures in TMEV-Infected B6 Mice

After intracerebral injection, 16 TMEV- and 8 PBS-injected mice were continuously VEEG-monitored for 14 days (Fig. 1A). Consistent with previous findings (8, 9), TMEV-injected mice exhibited mostly secondarily generalized behavioral seizures (Racine stage 3–5) after inoculation. Typical seizures consisted of electrographic preictal spiking associated with sudden immobility in a normally behaving animal or abrupt awakening if the mouse was sleeping (Fig. 1B). Spiking was followed by gradually evolving high-frequency discharges associated with forelimb and hindlimb clonus progressing to repetitive rearing and falling (complete loss of postural control). Electroencephalogram recordings show postictal suppression after the seizure, followed by prolonged periods of postictal spiking. During this time, the mouse displayed lethargy, excessive grooming, and/or wild running.

Of the 16 TMEV-injected mice, 12 (75%) exhibited Racine seizures, with an average frequency of 14.5 ± 10.5 (mean ± SD) seizures per animal during the entire recording period (Fig. 2A). Previous studies found that approximately 50% of infected mice had seizures; however, animals were only visually observed for a very limited period, and VEEG studies were not conducted (8, 9). For these reasons, we believe the proportion of animals displaying spontaneous seizures with continuous VEEG more accurately reflects the incidence of short-term symptomatic seizures. However, because focal hippocampal seizures may be undetected by the surface cortical electrodes used, this may still be an underestimate of the true seizure frequency. Thus, a minimum of 75% of TMEV-infected mice exhibited short-term seizures.

Seizures occurred between 3 and 7 days postinfection (dpi); the majority occurred at 4 and 5 dpi (Fig. 2B). On 3 dpi, approximately half of all seizures were partial stage 2 to 3 seizures, with an average duration of 24 ± 13 seconds. Seizures became progressively more severe, with most mice displaying secondarily generalized stage 4 to 5 seizures by 5 dpi. Furthermore, the average seizure duration increased significantly from 24 ± 13 to 50 ± 17 seconds (mean ± SD; p < 0.001; one-way ANOVA with Tukey post hoc). Although seizure frequency declined, the severity remained elevated until 7 dpi, at which time seizure activity stopped abruptly (Figs. 2C, D).

During the second week of monitoring (8–14 dpi), mice did not display any seizures, although there was intermittent spiking

FIGURE 2. Short-term spontaneous encephalitic seizures in Thie"{l}er’s murine encephalomyelitis virus (TMEV)–injected mice. (A) 75% of TMEV-injected mice displayed 1 or more convulsive electrographic seizure during the first week postinjection (PI). No seizures were present during the second week. Phosphate-buffered saline–injected control mice never displayed seizures. (B) The average number of seizures in the TMEV-injected mice that displayed seizures was plotted over time to illustrate the timeframe of seizure occurrence as well as seizure frequency over time. Seizures invariably occurred between days 3 and 7 PI, abruptly terminating by day 8 PI. (C) Most seizures were secondarily generalized stage 4/5, with partial stage 2/3 seizures occurring mostly at the beginning and at the end of the seizure period. (D) The seizure duration on days 5 and 6 PI was significantly longer compared with other days during the seizure period (p < 0.01 by ANOVA with Tukey post tests; n > 8 mice per time point, 12 mice total).
observed on the EEG (data not shown). No PBS-injected mouse displayed seizures or interictal spiking.

Long-Term Recurrent Seizures After TMEV Infection

After TMEV inoculation, mice were visually observed 2 h/d for 2 weeks for the development of short-term symptomatic seizures (Fig. 3A); 60% of these mice displayed stage 4 to 5 behavioral seizures at 3 to 7 dpi. Mice observed to have at least 1 seizure during this period were used for subsequent long-term studies. At 7 weeks PI, mice were surgically implanted with recording electrodes and allowed to recover.

The first set of long-term VEEG monitoring experiments commenced at 8 weeks PI, and these mice were monitored continuously for 1 week. Of the 14 TMEV-infected mice monitored, 8 exhibited behavioral seizures (57%) and all 14 displayed epileptiform activity (Table). Behavioral seizures observed during the long-term period were similar to those observed during short-term monitoring period (compare Fig. 1B with Fig. 3B) and were characterized by high-frequency electrographic discharges associated with stage 4 or 5 activity. Epileptiform activity consisted of spiking correlated with behavioral arrest (Fig. 3B-3) and/or rhythmic spiking with no behavioral correlate (Fig. 3B-4).

FIGURE 3. Chronology of procedures and representative seizures in epileptic Theiler’s murine encephalomyelitis virus (TMEV) mice. (A) Timeline of procedures. Animals were monitored with long-term video-electroencephalogram (VEEG) at 3 different time points during the long-term period, that is, 2 months postinjection (PI) for 1 week (n=14), 4 months PI for 1 month (n = 7), and 7 months PI for 1 month (n = 5). (B) Representative EEG traces showing baseline activity in a control (PBS) mouse (top trace) compared with high-frequency, high-amplitude, and rhythmic activity recorded during a stage 5 seizure at 4 months PI. In the expanded traces [1–4], epileptiform activity associated with behavioral arrest [3] or no behavioral arrest [4] is present in TMEV mice but not in PBS mice [1]. Activity observed during a stage 5 seizure is expanded for comparison [2].

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Because of the relatively low behavioral seizure frequency (2.1 ± 1.8 seizures per animal per week), we hypothesized that if we monitored for a longer period we would observe a greater percentage of mice displaying seizures. It was also possible that the development of an epileptic circuit was progressive, leading to an increased seizure frequency as the mice aged. Therefore, mice were continuously monitored for 1 month at 2 months postinjection (PI) and for 4 weeks at 4 and 7 months PI. All mice that retained their recording electrodes, regardless of whether they displayed seizures during the first long-term monitoring time point (2 months PI) or not, were again monitored at 4 months PI and/or 7 months PI.

*Seizures were generally stage 4/5.

Although the mean seizure frequency seemed to decrease with time, differences between the time points and differences in mean seizure duration over time were not significant (p > 0.05; ANOVA).

### TABLE. Long-Term Behavioral and Electrographic Seizure Activity in Theiler’s Mouse Encephalomyelitis Virus–Infected Mice

<table>
<thead>
<tr>
<th></th>
<th>2 mo PI</th>
<th>4 mo PI</th>
<th>7 mo PI</th>
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</thead>
<tbody>
<tr>
<td>No. mice with seizures*</td>
<td>64% (9/14)</td>
<td>57% (4/7)</td>
<td>40% (2/5)</td>
</tr>
<tr>
<td>No. mice with epileptiform activity</td>
<td>100% (14/14)</td>
<td>100% (7/7)</td>
<td>100% (5/5)</td>
</tr>
<tr>
<td>Seizure frequency, mean ± SD, seizure per mouse per week†</td>
<td>2.1 ± 1.8</td>
<td>0.75 ± 0</td>
<td>0.37 ± 0.1</td>
</tr>
<tr>
<td>Seizure duration, mean ± SD, s†</td>
<td>53.4 ± 27.1</td>
<td>45.8 ± 17.2</td>
<td>66.2 ± 27.0</td>
</tr>
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</table>

Mice were monitored continuously for 1 week at 2 months postinjection (PI) and for 4 weeks at 4 and 7 months PI. All mice that retained their recording electrodes, regardless of whether they displayed seizures during the first long-term monitoring time point (2 months PI) or not, were again monitored at 4 months PI and/or 7 months PI.

*Seizures were generally stage 4/5.

†Although the mean seizure frequency seemed to decrease with time, differences between the time points and differences in mean seizure duration over time were not significant (p > 0.05; ANOVA).
was 1.4 ± 1.6 per mouse per week, with an average duration of 54 ± 25.5 seconds (Table). None of the PBS-injected mice (n = 9) displayed seizures or epileptiform activity.

Hippocampal Sclerosis After TMEV Infection

Brain sections from TMEV-inoculated mice at 2 to 8 months PI were stained with cresyl violet. An investigator blinded to treatment protocols qualitatively assessed these sections. This analysis revealed severe hippocampal damage characterized by cell loss in the CA1 and CA2 pyramidal cell layer and corresponding enlargement of the lateral ventricles (Figs. 4A, B). This damage was further confirmed by measuring ventricular and hippocampal areas using ImageJ software (National Institutes of Health; Fig. 4C). Both the ipsilateral and contralateral sides of each section were qualitatively analyzed (4–8 sections per animal). Because there did not seem to be any overt differences, these results were combined for quantification purposes. In 7 TMEV-injected mice, the lateral ventricle areas were significantly enlarged (0.92 ± 0.11 mm²) versus the PBS mice (0.44 ± 0.057 mm²; p < 0.001 by unpaired t test). Furthermore, the hippocampi were significantly smaller in the TMEV mice (1.7 ± 0.081 mm²) versus those in the PBS mice (2.3 ± 0.11 mm²; p < 0.001 by unpaired t test).

NeuN staining was used to confirm neuronal cell loss in the pyramidal cell layer. There was considerable cell loss and collapse of the alveus evident at 2 months PI (Fig. 4B), but by 4 months, PI the hippocampus seemed to be reconnected to the cortex by scar tissue (Fig. 4E). There were varying degrees of pyramidal cell layer damage in TMEV-injected mice. For example, in some mice, there was thinning of the CA1 and CA2 region into a single cell layer with sparing of the CA3 area, whereas in others there was complete degeneration of the region. In contrast to the CA1, CA2, and CA3 regions, the dentate gyrus and CA4 remained relatively intact in all mice (Figs. 4B, E).

Astrogliosis is present in areas of severe neural cell loss in patients with hippocampal sclerosis (17). To determine whether glial scarring was present in the pyramidal region, we performed immunofluorescence assays for GFAP. There was greater GFAP immunoreactivity in CA1 and CA2 pyramidal regions in 9 TMEV mice (4–6 months PI) versus controls (n = 7; Fig. 5). Reactive astrocytes showed increased GFAP immunoreactivity in the somata and primary processes (Fig. 5F). Variable astrogliosis was also found in other brain areas, including thalamic and amygdaloid nuclei and the piriform and entorhinal cortices.

Figure 5. Long-term astrogliosis in sclerotic hippocampal pyramidal cell layer of Theiler’s murine encephalomyelitis virus (TMEV)-injected mice. (A–F) The top row shows hippocampal sketches with red rectangles indicating the region shown in the images below. The middle row (A–C) shows CA1 (A) and CA2/3 (B, C) sections from a control (PBS)-injected mouse. The section in (C) is a magnified region of B (note scale bar in F). The bottom row (D–F) shows corresponding sections from a TMEV-injected mouse. Note the increased glial fibrillary acidic protein (GFAP) immunoreactivity in the somata and proximal processes of individual reactive astrocytes (indicated by arrow in F) compared with GFAP+ astrocytes in control section in (C). Scale bar = 100 µm.
Absence of Mossy Fiber Sprouting

The presence of Timm stain reaction product in the supragranular and/or molecular layer of the dentate gyrus is used as a marker for mossy fiber sprouting (15). Timm staining is a histochemical marker of zinc and other heavy transition metals, and the reaction product is restricted to the dentate hilar region in naive tissue. The amount of mossy fiber reorganization was assessed throughout the hippocampus of TMEV-infected (n = 13) and PBS-injected mice (n = 9) at 4 to 7 months PI. A total of 102 sections from PBS mice and 149 sections from TMEV mice (ipsilateral and contralateral) were analyzed by an individual blinded to the experimental treatment. Phosphate-buffered saline–injected mice exhibited intense Timm staining extending from the hilus to the proximal dendrites of CA3 pyramidal cells (Fig. 6A). No dark reaction product was detected in the molecular layer of the dentate gyrus (mean Timm score = 0). Timm staining in TMEV-injected mice was similar to that observed in control mice, with the reaction product concentrated in the hilus and projecting to the CA3 region (Fig. 6B). One TMEV-injected mouse showed reaction product projecting through the hippocampal granule cell layer and into the molecular layer (Fig. 6C). The mean Timm score for TMEV mice was 0.2 ± 0.2 (mean ± SEM). Thus, we did not observe any significant mossy fiber reorganization in TMEV mice at a time point when seizures were observed.

**DISCUSSION**

Fundamental issues regarding the role of CNS infections in the pathogenesis of epilepsy syndromes are unresolved. We describe experiments establishing TMEV infection of B6 mice as a novel model of epilepsy that may prove useful for elucidating the effects of CNS infection on brain hyperexcitability. Several important conclusions can be drawn from these findings. First, TMEV infection causes short-term encephalitic seizures and ultimately the development of epilepsy in B6 mice. Although short-term seizures after CNS infections have been reported in other viral models (5–7), the development of epilepsy after a short-term infection has not previously been demonstrated in an animal model. Second, we provide evidence that TMEV infection leads to hippocampal sclerosis consisting of both neuronal cell loss and astrogliosis in B6 mice. Third, there are several transgenic lines available on the B6 background, thereby further increasing the potential experimental utility of this model. Fourth, certain aspects of the encephalitic and infectious processes in TMEV-infected B6 mice mimic human infection-induced epilepsy, including short-term symptomatic seizures, latent period, interictal events, and long-term spontaneous behavioral seizures. Thus, TMEV infection of B6 mice provides a small tractable animal model to evaluate the mechanisms underlying short-term encephalitic seizures, epileptogenesis, long-term seizures, and associated histopathologic alterations. Moreover, postencephalitic epilepsy is often pharmacoresistant to existing antiepileptic drugs; thus, the development of a mouse model of viral-induced epilepsy provides a platform for evaluating new therapies for epilepsy.

Continuous VEEG monitoring studies indicated that 75% of TMEV-infected mice exhibit seizures during short-term TMEV infection. The narrow temporal window (3–7 dpi) during which short-term seizures occurred was notable. This occurrence may be related to the underlying processes such as viral propagation and/or accompanying inflammatory responses. Studies in TMEV-infected abstracter cultures show that at 3 dpi, infected B6 astrocytes produce significantly increased levels of cytokines, chemokines, and cell adhesion molecules, including interleukin-6 and tumor necrosis factor (TNF) (18). Transgenic mice with long-term interleukin-6 production develop hippocampal neurodegeneration, hyperexcitability, and spontaneous seizures (19). Although the effect of TNF on seizures is controversial (20), seizures and cortical injury in a rat model of bacterial meningitis were attenuated by reducing the soluble form of TNF, suggesting a proconvulsive role for it in that model (21). Previous studies in our model show that at 5 to 7 dpi, TGF-β is upregulated in the hippocampus of TMEV-infected mice that exhibit short-term symptomatic seizures (8). Other studies have shown that exogenous application (intracerebroventricular injection) of TGF-β suppressed kainic acid–induced seizures and prevented associated neuronal cell loss (22). These findings suggest that the temporal profile of the immune response during the short-term infection

**FIGURE 6.** Timm- and cresyl violet–stained hippocampal sections of control (PBS)-injected and Theiler’s murine encephalomyelitis virus (TMEV)–injected mice 8 months after injections. (A, B) Representative sections from a PBS-injected mouse (A) and a TMEV-injected mouse (B) show an absence of reaction product in the granule cell layer (g) and molecular layer (m) of the dentate gyrus, corresponding to a score of 0. In both PBS-injected (n = 9) and TMEV-injected (n = 13) mice, most animals had a score of zero. (C) In 1 epileptic TMEV-injected mouse, 3 of 8 sections examined showed slight but detectable Timm staining in the granule cell layer and molecular layers of the ipsilateral dentate gyrus (arrows), corresponding to a score of 2. Scale bar = 200 μm.
may be critical for seizure generation and termination. Indeed, we recently demonstrated that cytokines may be playing an important role in the short-term seizures that are observed after infection (23). Thus, the TMEV model will provide insight into the role of inflammatory cytokines in the pathophysiology of virus-induced epilepsy.

Patients with acquired epilepsy associated with a known initial precipitating injury often have a latent period between the initial insult and the first spontaneous seizure. Studies in animal models indicate numerous alterations in molecular and cellular components of glial and neural networks suggesting that the latent period coincides with epileptogenesis (24). Our studies show that after viral clearance and a latent period of at least 1 week (because no seizures were ever observed with EEG monitoring during the second week PI), 65% of TMEV mice that displayed short-term symptomatic seizures subsequently develop epilepsy. Further studies are needed to delineate the precise duration of the latent period because this may prove to be a critical window for investigating epileptogenesis and therapeutic interventions. It must also be emphasized that it is unlikely that viral persistence is involved in the long-term epilepsy observed in this model because numerous studies have shown that the virus is rapidly cleared from the CNS, that is, within 2 weeks after intracranial inoculation (9, 11, 25).

Up to 60% of patients with medically refractory temporal lobe epilepsy (TLE) have the associated finding of mesial temporal sclerosis, Ammon horn sclerosis, or simply hippocampal sclerosis (26). Hippocampal sclerosis is characterized by neuronal loss and astrogliosis in the hippocampal pyramidal cell layer (27). Successful surgical outcomes with respect to seizure termination have been achieved in more than 90% of patients with TLE associated with hippocampal sclerosis, suggesting that the sclerotic region is the main epileptogenic focus in this patient population (28). A retrospective study of patients who developed adult-onset TLE suggest that up to half of such patients may have developed epilepsy as a consequence of limbic encephalitis (29).

Studies in humans and animal models have indicated several morphologic and molecular changes in reactive astrocytes in sclerotic hippocampi, including a reduced ability to remove extracellular potassium in response to neuronal activity (30), increased glutamate release (31), and sustained activation of inflammatory signaling pathways (32). These alterations in molecular signaling of astrocytes would be expected to increase excitability in the surrounding neuronal tissue and contribute to seizure generation. Sclerotic hippocampi from TMEV-infected mice exhibit prominent reactive gliosis with notable alterations in astrocyte morphology. Electrophysiologic and further molecular studies would be useful for further assessing glial changes in these mice.

Hilar neuron cell death has been observed in some patients with TLE (33) and in many animal models (16, 34). Loss of hilar neurons is usually associated with mossy fiber sprouting. Interestingly, neither of these features were apparent in our TMEV model. This observation is not entirely unusual; for example, kainate-induced status epilepticus and amygdala kindling causes severe seizures in immature rats without corresponding detectable synaptic rearrangement in the dentate gyrus (35). Another possibility is that sprouting may occur at later time points PI that were not assessed in the present study. The differences in hippocampal pathology further support the idea that there may be fundamental differences between chemical-induced seizure models of adult-onset epilepsy and this infection-induced model. There is a clear need for additional longitudinal studies that would directly compare the pathology, latency to spontaneous seizures, and severity of seizures after TMEV infection to other rodent models of TLE. Until such a study has been conducted using the same rodent species and/or strain, direct comparisons between our TMEV model with other models of acquired epilepsy (e.g. poststatus epilepticus, traumatic brain injury, hypoxia, hypoxic-ischemia, or hyperthermia) would be premature.

In conclusion, the previous lack of a tractable animal model for assessing the role of CNS infection in epilepsy has made experimental hypothesis testing difficult. The novel model described here will allow further evaluation of these events. Importantly, the development of infection-induced epilepsy in the B6 strain of mice is fortuitous because this strain is most commonly used for genetic manipulations. Furthermore, the presence of extensive neuronal cell loss and glial scarring in the hippocampus of TMEV-injected B6 mice may have significant implications in these studies. Indeed, Buenz et al previously demonstrated that TMEV-infected B6 mice have significant learning and memory deficits that correlate with the extent of hippocampal neuronal cell loss after TMEV infection (36). In addition to confirming this damage, we also demonstrate epileptic seizures in TMEV mice. The correlation between cognition and epilepsy is an important avenue that requires further investigation. Thus, this model provides a basic framework for conducting preclinical work aimed at delineating the mechanisms and designing therapeutic interventions for neurologic deficits involving inflammation, demyelination, memory and cognitive impairments, network hyperexcitability, and pharmacoresistant epilepsy.

**REFERENCES**


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