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Mouse Models of Multiple Sclerosis: Experimental Autoimmune Encephalomyelitis and Theiler's Virus-Induced Demyelinating Disease

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Abstract

Experimental autoimmune encephalomyelitis (EAE) and Theiler's Murine Encephalitis Virus-Induced Demyelinating Disease (TMEV-IDD) are two clinically relevant murine models of multiple sclerosis (MS). Like MS, both are characterized by mononuclear cell infiltration into the CNS and demyelination. EAE is induced by either the administration of myelin protein or peptide in adjuvant or by the adoptive transfer of encephalitogenic T cell blasts into naïve recipients. The relative merits of each of these protocols are compared. Depending on the type of question being asked, different mouse strains and peptides are used. Different disease courses are observed with different strains and different peptides in active EAE. These variations are also addressed. Additionally, issues relevant to clinical grading of EAE in mice are discussed. In addition to EAE induction, useful references for other disease indicators such as DTH, in vitro proliferation, and immunohistochemistry are provided. TMEV-IDD is a useful model for understanding the possible viral etiology of MS. This section provides detailed information on the preparation of viral stocks and subsequent intracerebral infection of mice. Additionally, virus plaque assay and clinical disease assessment are discussed. Recently, recombinant TMEV strains have been created for the study of molecular mimicry which incorporate various 30 amino acid myelin epitopes within the leader region of TMEV.

Keywords

Multiple sclerosis; Experimental autoimmune encephalomyelitis; EAE; Emulsion; Active induction; Adoptive transfer; T cell blasts; Encephalitogenic; Neurodegeneration; Theiler's murine encephalomyelitis virus-induced demyelinating disease; PLP; MOG; Myelin; MBP; VP2; VP3; Relapsing–remitting; Epitope spreading

1. Introduction

Mouse models of demyelinating diseases have been useful in both the demonstration of T cell-mediated demyelination and in the characterization of the pathogenesis of immune-mediated demyelinating disease. This chapter describes the methods for inducing and characterizing two models of demyelinating disease: experimental autoimmune encephalomyelitis (EAE) and Theiler's murine encephalomyelitis virus-induced demyelinating disease (TMEV-IDD).

EAE is a CD4⁺ T cell-mediated, demyelinating autoimmune disease of the CNS that is characterized by mononuclear cell infiltration. EAE serves a useful animal model for multiple sclerosis (MS), since many of the pathologies observed in the CNS of mice with EAE bear strong similarity to those found in the CNS of MS patients (1–9). In both EAE and MS, the white matter of the CNS presents with demyelinating lesions associated with infiltrating T cells, macrophages, and B cells (10–15). In addition, foam cell-like macrophages containing phagocytosed hydrophobic myelin debris have been demonstrated

within active lesions (16–18). Ascending hind limb paralysis (described in Subheading 3.1.3 , below) is associated with inflammation and demyelination of axonal tracks. Finally, oligoclonal IgG can be found in the CSF of both EAE mice and MS patients (2, 7, 19).

There are two widely used methods for inducing EAE in mice: active induction by immunization with myelin antigens and passive induction by the adoptive transfer of pre-activated myelin-specific T cells into naïve mice. In active EAE, peripheral immunization of mice with myelin antigen(s) results in the breakdown of peripheral tolerance and the activation of myelin antigen-specific T cells in the secondary lymphoid organs. Following activation, myelin-specific T cells proliferate and differentiate into effector cells, allowing egress from the secondary lymphoid organs. The expression of integrins by effector T cells enables them to cross the blood–brain barrier (20), where they are reactivated by CNS-resident APCs presenting myelin antigens (21). Reactivation leads to the expression of pro-inflammatory cytokines by the effector T cells (IFN- γ , IL-17, GM-CSF, and TNF- α), some of which can directly injure nervous tissue. In addition, chemokine production by the pathogenic T cells induces recruitment of nonspecific cellular effectors such as $\gamma\delta$ T cells, monocytes, macrophages, and neutrophils into the CNS (22, 23). Activation of these inflammatory cells and the bystander damage they mediate are largely responsible for destruction of the myelin-sheathed axonal tracts and the formation of lesions.

The effector phase of EAE can be directly induced by the adoptive transfer of activated, myelin-specific Th1 or Th17 cells from immunized donors into naïve syngeneic recipients (24–26). Although the clinical features of disease induced by passive EAE are identical to those induced by active EAE, and more reagents are required, passive EAE has numerous advantages over active induction since (a) the day of adoptive transfer serves as a definitive point of introducing encephalitogenic T cells to the recipient mice; (b) there is no antigen depot to present leading to continuous de novo activation of naïve T cells; and (c) it can be used to track encephalitogenic T cells in vivo; (d) to study CNS infiltration, and (e) isolate antigen-specific T cells from the CNS (27, 28). Passive EAE induction is a valuable tool for delineating the relative contributions of T helper subsets in disease, and it is considered to be a more direct way of characterizing T cell effector function in the CNS. In the SJL/J mouse, both active induction and adoptive transfer of disease typically take a relapsing–remitting form, while the C57BL/6 mouse displays chronic–progressive disease following active or passive EAE induction.

TMEV-IDD has been defined as a mouse model for human multiple sclerosis (29, 30). TMEV is a natural mouse pathogen that belongs to the cardiovirus group of the Picornaviridae family (31, 32), and is composed of a single, positive-strand RNA genome surrounded by a capsid containing viral proteins, VP1, VP2, and VP3. TMEV is divided into two subgroups based on the pathogenesis of the viruses. The first subgroup, which includes GDVII, is highly virulent and induces fatal encephalitis in infected mice. The second group, which is defined as the Theiler's original subgroup, includes Daniels (DA) and BeAn 8386 strains that have low virulence and do not induce severe encephalitis, but do establish persistent infections of the CNS associated with immune-mediated demyelination (33).

TMEV-IDD is an immune-mediated demyelinating disease dependent on persistent virus infection of the macrophages, microglia, and astrocytes within the CNS (34, 35). TMEV-IDD is associated with a mononuclear cell infiltrate consisting predominantly of CD4⁺ T cells, macrophages, and B cells. The chronic phase of TMEV-IDD is mediated by a PLP_{139–151}-specific CD4⁺ Th1 type T cell response that can be initially detected at approximately 45–55 days post-infection (36). As the disease progresses, epitope spreading leads to autoimmune responses to additional myelin antigens (37). The inflammation and demyelination observed in TMEV-IDD are similar to the pathological descriptions in MS

patients (38, 39). Importantly, epidemiological studies suggest a viral etiology for MS providing additional importance for TMEV-IDD as a relevant model for multiple sclerosis (40, 41). Infection of SJL/J mice with the BeAn strain has been directly associated with the development of a chronic–progressive demyelinating disease arising approximately 30–35 days post-infection characterized by spastic hind limb paralysis and primary demyelination (42).

In this article, we address the basic methods for inducing both active and adoptive transfer of EAE and the induction of TMEV-IDD. While different mouse strains have various susceptibilities to both models of disease, we focus primarily on the induction in the most commonly used strains of mice, the SJL/J and C57BL/6. Additionally, the induction of TMEV-IDD is described using the BeAn strain of TMEV in the SJL/J mouse. Mouse strain susceptibilities and variation in disease are discussed in the Notes section 5.

2. Materials

2.1. Induction of Active EAE

1. SJL/J or C57BL/6 mice (Harlan Laboratories).
2. Encephalitogenic protein or peptide or spinal cord homogenate.
3. Phosphate-Buffered Saline (PBS).
4. *Mycobacterium tuberculosis*, H37 RA (Difco).
5. Incomplete Freund's Adjuvant (Bacto).
6. 3-Way nylon stopcock (Luer connector) (Kontes Glass Co) or Sorvall Omni-Mixer (Dupont Instruments).
7. 5-ml snap cap tubes (Falcon).
8. Small animal clippers (Golden A5, Oster).
9. 18 and 25 gauge needles (Becton Dickinson).
10. Pertussis toxin (List Biological Labs).
11. Carbol fuchsin dye.
12. Ear tags (Gey band and tag).

2.2. Induction of Passive EAE

1. SJL/J or C57BL/6 mice (Harlan Laboratories).
2. Balanced salt solution (BSS).
3. Dulbecco's modified Eagle's medium (DMEM).
4. Heat-inactivated fetal calf serum (FCS).
5. 200 mM L-glutamine.
6. 5.5 mM β 2-mercaptoethanol.
7. 1,000 U/ml penicillin, 1,000 μ g/ml streptomycin.
8. Light microscope and hemocytometer.
9. 75 cm² sterile tissue culture flasks.
10. Myelin antigen or myelin peptide.

11. 10 ml sterile pipette.
12. 100 gauge sterile wire mesh and 90 mm sterile Petri dishes.
13. 3 and 10 ml syringes.
14. 25 gauge needle.

2.3. Induction of TMEV-IDD

1. DMEM (Sigma).
2. FCS (Sigma).
3. Tryptose phosphate broth (Sigma).
4. Antibiotic–antimycotic (Gibco BRL).
5. BHK-21 cells (ATCC).
6. 25-, 75-, 162-cm² tissue culture flasks (Corning).
7. Versene 1:5,000 (Gibco BRL) or Trypsin–EDTA (Sigma).
8. Centrifuge tubes.
9. Polyethylene glycol (PEG) (Sigma).
10. Tris base (Sigma).
11. Sodium chloride, NaCl (Sigma).
12. Sodium dodecyl sulfate, SDS (Sigma).
13. Sucrose (Sigma).
14. 21-, 23-, and 27-gauge needles.
15. Cesium sulfate, Cs₂SO₄ (Sigma).
16. PBS.
17. 60 mm tissue culture dishes (Nunc).
18. Noble agar (Sigma).
19. Penicillin/streptomycin (Life technologies).
20. Crystal violet (Sigma).
21. ClaI restriction enzyme (Promega).
22. DH5α max efficient *E. coli* (Invitrogen).
23. Sp6/T7 in vitro transcription kit (Roche).
24. Lipofectin reagent (Gibco BRL).
25. SJL/J mice (Harlan Laboratories).

3. Methods

3.1. Induction of Active and Passive EAE

3.1.1. Induction of Active EAE Disease—EAE can be induced in susceptible strains of mice by using proteolipid protein (PLP), myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), or peptides corresponding to the encephalitogenic

portions of these proteins. Peptides (>98 % purity based on mass spectrophotometry) or spinal cord homogenate to be used in priming are first dissolved in PBS and irradiated at 6,000 rads for sterilization purposes. For peptides that are insoluble in PBS (pH 7.0), the pH can be raised until the peptide dissolves. The pH can then be lowered to physiologic levels; however, pH has little influence on disease induction with peptide in CFA. Peptide should be diluted in PBS to a concentration of 1 mg/ml if inducing disease with PLP_{139–151} in the SJL/J mouse. MOG_{35–55}-induced disease in the C57BL/6 mouse requires a concentration of 4 mg/ml in PBS as do all other peptides used to induce C57BL/6 or SJL/J disease. An equal volume of peptide/PBS is added to complete Freund's adjuvant (containing 4 mg/ml—desiccated *M. tuberculosis*, H37 RA in Incomplete Freund's Adjuvant). This is then thoroughly mixed to form a thick peptide/CFA emulsion. For small volumes, the emulsion can be prepared directly between two 1 ml tuberculin syringes using a 3-way stopcock with a Luer connector. For larger volumes, the emulsion can be prepared using a mechanical mixer with the emulsion on ice. Emulsion should be removed from the mixer with a standard lab spatula and placed in a 5-ml snap cap tube. The emulsion is then gently centrifuged. The emulsion should be loaded into 1 ml tuberculin syringes using an 18 gauge needle taking care not to introduce air bubbles into the syringe. The 18 gauge needle is replaced with a 27 gauge needle for immunization. Table 1 provides a comprehensive list of different peptides of PLP, MBP, and MOG proteins which can be used to initiate disease in different inbred mouse strains.

The backs of animals to be primed are shaved using small animal clippers. Ideally, animals receive approximately 100 µl of emulsion subcutaneously divided equally across three sites on the dorsal flank (on each hip and one along the midline of the back between the shoulders) using a 27 gauge needle. If inducing EAE in the C57BL/6 strain or R-EAE in the SJL/J strain with MBP or MOG antigens, 200 ng of pertussis toxin must be administered intraperitoneally (i.p.) on days 0 and 2 relative to immunization. As necessary, animals should be marked for grading purposes. White and light brown mice can be marked with a red dye (carbol fuchsin). We routinely mark mice with large spots on the head, midback, base of tail, and left and right sides. For black mice, one can mark the tails with permanent markers, e.g., Sharpie, and reapply as necessary (one, two, three stripes, etc.) or use numbered ear tags.

3.1.2. Induction of Passive EAE—Eight- to twelve-week-old mice are immunized as described above for active induction of EAE (24, 25). In some cases, such as the adoptive transfer of myelin-specific transgenic T cells and encephalitogenic T cell lines maintained by in vitro passage, this step is not necessary (see Notes). Once immunized, the mice are left for 7–14 days, as described for different models in Notes. The ratio of donor mice to recipient mice varies according to how many activated cells are required for adoptive transfer (see Table 2). Typically, for induction of PLP_{139–151}-induced disease in the SJL/J mouse, one donor mouse for two recipient mice is usually sufficient. In the C57BL/6 model, typically the ratio is one to one. In cases where large numbers of cells are to be transferred, higher ratios are required.

In models where donor mice are immunized (see Notes), inguinal, axillary, and brachial lymph nodes are pooled from primed mice and placed in BSS containing 5 % FCS. Cell suspensions should be kept on ice at all times. The pooled lymph nodes are then placed onto a sterile 100 gauge wire mesh in a 90 mm Petri dish. Using the plunger of a sterile 10 ml syringe, the lymph nodes are crushed to produce a single-cell suspension. The cell suspension is centrifuged at 300 g for 5 min at 4 °C in a sterile 50 ml conical centrifuge tube. The pellet is resuspended in fresh 5 % BSS by vigorous manual agitation or repeated pipetting using a sterile 10 ml pipette. The cells are washed once more with 5 % BSS and again pelleted as previously described. After centrifugation, the cells are resuspended in

complete culture medium (DMEM containing 10 % FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin, and 50 µM 2-mercaptoethanol). Three milliliter of culture medium are added for each donor mouse used to obtain lymph node cells. Alternatively, complete HL-1 (serum free) can be used as culture medium in place of DMEM.

Primed LN cells are resuspended at a final concentration of 8×10^6 cells/ml in complete DMEM medium. Thirty milliliter (2.4×10^8 total cells) are placed into a sterile 75 cm² tissue culture flask. Myelin protein or peptide antigen is added to the lymph node suspension at the concentrations indicated in Table 2. Cells are cultured for 3–4 days (37 °C, 100 % humidity, and 5 % CO₂) for the time periods indicated in Table 2 for the various models. In transfer systems where T cell lines or naïve transgenic T cells (B10. PL—MBP_{Ac1-11}-specific transgenic T cells) are to be reactivated or activated, respectively, different protocols are followed and can be obtained from the relevant references (43, 44). In these systems, addition of recombinant IL-12 to the culture medium is sometimes required (see Table 2) for efficient induction of clinical disease in naïve recipient mice. In some cases, neutralizing antibody to IL-4 has also been used (43). Culturing lymphocytes in the presence of IL-23 rather than IL-12 may be desirable if the investigator is attempting to skew myelin-specific T cells towards a Th17 phenotype (23, 45).

After 3–4 days of in vitro culture, the cells are resuspended by repeated pipetting and pelleted in a 50 ml conical tube. Cells are washed twice with BSS and resuspended in approximately 10 ml of BSS for each 30 ml tissue culture volume. This blast preparation consists mainly of CD4⁺ T cells. If required, CD8⁺ T cells and any remaining B cells and macrophages can be depleted using magnetic bead separation techniques or other standard depletion methods. Viable cell counts of the number of T cell blasts are determined by counting on a hemocytometer. Both the total numbers of cells and the total numbers of T cell blasts are determined. Unstimulated T cells appear small, quite regular in shape with a cytoplasm relatively clear, compared to the large, often irregular and granular appearance of T cell blasts. Typically, in the SJL/J transfer system, blasts account for 30–40 % of total cells, although use of complete HL-1 will yield a blast percentage of 20–30 % of total cells. In other systems, the percentage may be lower and in transgenic systems, higher. Cells are resuspended at a concentration of 2×10^6 – 1.2×10^8 T cell blasts/ml in PBS, depending on the adoptive transfer system and the number of blasts transferred (see Table 2).

The T cell blasts, derived above, can be injected into naïve syngeneic recipient either intraperitoneally or intravenously (i.v.) to induce effective clinical disease. However, i.v. injection is typically more effective, with clinical disease developing faster than delivery via i.p. injection. Usually the required numbers of cells are injected in a volume of 0.2–0.5 ml using a disposable 1 ml tuberculin syringe and a 25 or 27 gauge needle. Adoptive transfer of MOG₃₅₋₅₅-specific blasts into naïve C57BL/6 mice must be accompanied by i.p. injection of 200 ng of pertussis toxin on days 0 and 2 relative to adoptive cell transfer.

3.1.3. Clinical Grading of Active and Passive EAE—Following priming, mice should be monitored every other day for the development of disease. The appearance of EAE disease induced by active immunization varies considerably based on mouse strain and peptide used. For most strains and peptides, disease appears between the second and fourth week following priming. The disease is characterized by an ascending hind limb paralysis that begins in the tail and spreads to involve the hind limbs and forelimbs. The disease is graded on a 0–5 scale, though depending on strain and peptide, mice do not always reach the higher disease grades before disease resolution or disease plateau. *Grade 0*: there is no observable difference from naïve animals. *Grade 1*: assigned to mice that have lost tail tonicity or show hind limb weakness (*but not both*). Loss of tail tonicity is judged in mice

that when held aloft by the base of the tail show sagging of the tail and the tail cannot be lifted. Additionally, the tip of the tail fails to curl. Hind limb weakness is defined by the objective criterion that when placed on the wire screen of the cage, the animal's legs fall through as it tries to walk. A waddling gait can also be observed as the animal walks on a flat surface. The rear limbs are splayed and the rear posture lowered. *Grade 2*: assigned to mice that present *both* a limp tail and show hind limb weakness as defined above. *Grade 3*: assigned to mice that show partial hind limb paralysis defined as the ability of a mouse to move one or both hind limbs to some extent but not maintain posture or walk. *Grade 4*: assigned to mice that cannot move their hind limbs. The animal moves only by dragging itself with its front limbs. A spastic paralysis and atrophy of the hind limbs and lower body are often observed at this point. Mice at this stage are given food on the cage floor (that can be moistened), bottles with long sipper tubes, and daily injections of subcutaneous saline to prevent death by dehydration. *Grade 5*: assigned to the most severe end-stage assessment of EAE. These mice show a complete inability to move due to paralysis in all limbs. In addition, any animals that consistently show high grades and die (death by EAE) should be given a grade of five. Mice that reach this stage and are moribund with EAE should be sacrificed for humane reasons.

Histopathologically, the disease can be characterized by CD4⁺ T cell and F4/80⁺ (macrophage) inflammatory cell infiltrates that can be found in both diffuse and focal patterns. In most EAE models, the pattern of infiltrate tends to concentrate in the thoracic section of the spinal cord with less involvement in other regions of the cord or the brain. However, recent studies have revealed distinct differences in the histopathological features and infiltration profile induced by Th1 and Th17 cells (23, 46).

3.1.4. Clinical Disease Course—The first clinical episode is referred to as acute-phase disease which is preceded by pronounced weight loss. Mice will experience this acute episode for variable times depending on whether the disease is relapsing and remitting (R/R), monophasic, or chronic/progressive in nature. The point where disease reaches its highest score is referred to as the peak of acute disease. After the initial episode or a subsequent relapse, some strains of mice experience a recovery (remission). If the recovery lasts for at least 2 days and drops by at least one grade level, the recovery is deemed an authentic remission. These recoveries are observed in mice that show relapsing and remitting (SJL/J) and monophasic disease profiles (B10.PL). Mice that remit from the initial disease episode and recover fully or stabilize at a reduced disease score are referred to as monophasic, e.g., B10.PL primed with MBP_{Ac1-11}. Mice that have an acute disease that never shows a full grade reduction in disease are said to be prone to a chronic disease. This chronic disease is characterized by sustained priming antigen-specific T cell responses, e.g., C57BL/6 following MOG₃₅₋₅₅ priming.

In adoptive transfer EAE, clinical disease is evaluated using the same scale as for actively induced EAE. The type of disease, day of onset, and peak severity of disease depend on the system used (see Table 2). Onset to peak disease is rapid, necessitating daily evaluation of mouse clinical signs. Results are typically presented as mean clinical score of mouse groups $\pm$ standard error of the mean. Other critical measures include mean day of onset, mean peak score, and mean day of remission and relapse (the latter two being relevant to R/R disease). Typically, mice reach peak disease within a day or two after disease onset and remain at peak disease longer than in the active EAE induction (average 5 days versus 3 days). Disease incidence is normally greater than 90 %. Clinical disease may also be evaluated using a terminal evaluation of histopathology in which fixed and Epon embedded sections of spinal cord are stained with Toluidine blue, as described previously (47) (Fig. 1).

When choosing the strain of mice and clinical disease course to be studied, the investigator should take into consideration the advantages and disadvantages of each model. For example, the R-EAE disease course exhibited by SJL/J mice may be particularly useful for studies involving immunoregulation and epitope spreading since the mouse exhibits periods of remission as a result of self-regulation, and relapses associated with T cell responses spreading to other myelin epitopes. Although R-EAE recapitulates the most common clinical manifestation of MS, the chronic–progressive disease course exhibited by C57BL/6 mice is a popular model for EAE study due to the availability of transgenics and knockouts on the H-2^b background.

3.2. Induction of TMEV-IDD

3.2.1. TMEV Infecting Stock—Virus is produced in BHK-21 cells (ATCC). BHK-21 cells are grown in DMEM (Sigma) supplemented with 10 % FCS, 0.295 % tryptose phosphate broth (Sigma), 1.0 % gentamycin (Gibco BRL), and 1 % antimycotic–antibiotic (Gibco BRL). Cells are maintained in culture at 37 °C and 5 % CO₂ and grown to confluence. BHK-21 cells are removed from the flask by rinsing with versene (1:5,000) (Gibco BRL) or 1× Trypsin–EDTA Solution (Sigma), resuspended in complete medium (above), and seeded (1:10 split) in a new flask. These BHK-21 cells are grown to confluence (2–3 days), and washed with DMEM without serum or supplements.

Medium is removed from the cells, and TMEV, BeAn 8386 strain, is added at an MOI of 5 in a minimal volume of serum-free medium ensuring that the cell monolayer is covered with medium. The infected cells are incubated overnight at 33 °C at 5 % CO₂ until BHK-21 cells detach from the flask surface indicating lysis. The medium is transferred to a conical for centrifugation to pellet the cell debris. The cell lysate is removed and stored on ice, leaving a small volume on top of the pelleted cells. The pelleted cells are then sonicated using brief pulses to completely lyse the cells, and the resulting cell debris is again pelleted by centrifugation. The lysate is added to the stored lysate collected from the first spin and this is aliquoted into small volumes and stored at –70 °C. The titer of the infecting virus stock is determined by plaque assay (described below).

3.2.2. Purification of TMEV—Virus is produced in large stocks as described in Subheading 3.2.1 until the point of removing the supernatant from the infected cells. The supernatant is removed from the infected cells and the pH is adjusted with HCl to a pH below 7.0 and then frozen in bottles at –20 °C. The bottles are thawed in 37 °C shaking water bath without allowing the supernatant to become too warm. To each 500 ml of lysate, 14.5 g NaCl and 30 g PEG are added and the lysate is stirred overnight at 4 °C. The precipitated lysate is centrifuged at 7,000×g in a Sorvall HB-4 swinging bucket rotor for 45 min at 4 °C. The supernatant is discarded, and the pellet is immediately resuspended in 18 ml hypertonic TNE buffer (0.02 M Tris base, 0.5 M NaCl, 0.002 M EDTA). The resuspended pellets are sonicated to separate the virus from cellular debris and DNA. The pellets are pooled, warmed, and incubated with 1 ml 10 % SDS for each 9 ml of resuspended pellets for 30 min at 37 °C. The lysate is then centrifuged to remove any membranous debris. The supernatant is transferred to clear centrifuge tubes and overlaid onto 22 ml of a 35 % sucrose solution. The virus is pelleted through the sucrose by centrifuging in an SW28 rotor at 20,000 rpm for 20 h at room temperature.

Following the centrifugation, the pellet is resuspended in 2 ml hypertonic TNE buffer, and sonicated to remove clumps. The virus solution is then incubated with 0.1 ml 10 % SDS for each 1 ml solution for at least 10 min at 37 °C to remove the remaining membranous fractions. The virus solution is then clarified by centrifugation and overlaid with 2–3 ml of the resulting supernatant onto 20–70 % sucrose gradients poured in clear centrifuge tubes.

The gradients are centrifuged at 35,000 rpm for 3 h at room temperature. Following the centrifugation, a blue band containing the virus is visible by UV or halogen light about 2–3 cm from the bottom of the tube. The virus-containing band is collected by puncturing the side of the tube using a syringe fitted with a 21 gauge needle. The bands are pooled in new tubes up to a maximum volume of 1.5 ml per tube. 2.2 ml of 1 g/ml Cs₂SO₄ solution is then added to each tube. The tubes are filled with hypotonic TNE buffer, mixed thoroughly, and centrifuged at 40,000 rpm for 22 h at 4 °C.

The following day, the virus contained in a white band about 1 cm from the bottom of the tube is collected using a syringe fitted with 23 gauge needle. The virus-containing bands are pooled in a new centrifuge tube (2–3 ml per tube) and the tubes are then filled with hypotonic TNE buffer, mixed, and centrifuged at 35,000 rpm for 3 h at 5 °C. After the centrifugation, the virus pellet is resuspended in 0.2 ml PBS and incubated for 24 h at 4 °C. To increase virus yields, sonication of the suspension will help separate virus particles and remove virus adhering to the tube wall. Quantitate the virus by measuring the A_{280} and determine the amount of virus using the following equation: (average $A_{280} \times 10/35$) = mg virus. Purity is assessed by SDS-PAGE.

3.2.3. TMEV Plaque Assay—BHK-21 cells are cultured in 35 mm culture dishes (Thermo Fischer: Nunc, Rochester, NY) to 90 % confluence as described above. The BHK-21 cells are washed twice with serum-free DMEM. Dilutions of the virus stock or tissue homogenate are made in serum-free DMEM and 0.5 ml of each dilution is added to the BHK-21 cells in duplicate. The cells are incubated at room temperature for 1 h with occasional rocking of the dishes. Meanwhile, a 2 % solution of noble agar (Sigma) is autoclaved and maintained at 55 °C. A 1:1 solution of the 2 % noble agar and 2× DMEM supplemented with 2 % FCS and 2 % penicillin/streptomycin (Life technologies) is then prepared. After the 1-h incubation, 6 ml of the 1:1 agar:DMEM solution is added to each culture dish. The cells are then incubated at 33 °C and 5 % CO₂ for 5–6 days. The agar is removed from the dish, and the cells fixed with methanol and stained with a crystal violet solution (0.8 g crystal violet, 100 ml ethanol, 400 ml H₂O) for 5 min. Alternatively Formalin (Fischer) can be placed on top of the agar for 5 min to fix cells before the agar is removed and cells are stained with crystal violet as stated previously. The plate is then rinsed in a dish with water to remove the excess stain. Plaques are then counted and the number of plaque-forming units is calculated based on the dilution, volume of the dilution added to each culture dish, and weight of tissue used.

3.2.4. Construction of TMEV Containing Molecular Mimics of Myelin Peptides

—The cDNA for BeAn genome has been inserted into pGEM plasmid for molecular manipulations. A restriction enzyme site, ClaI, was inserted into the leader sequence of the BeAn genome along with a 23 amino acid deletion. Molecular mimic sequences for myelin epitopes as previously described (48) are inserted into the ClaI restriction site. PCR mutagenesis was conducted to insert ClaI sites flanking the sequence to be inserted into the virus genome. The mimic sequences are 30 amino acids in length to restore the deletion in the leader protein. The mimic sequence is ligated into the ClaI site in the BeAn cDNA, and DH5α *E. coli* are transformed with the ligated product to produce a BeAn cDNA containing the mimic sequence in the correct orientation. Next, in vitro transcription of the BeAn cDNA containing the mimic sequence is driven by an upstream T7 promoter using an Sp6/T7 in vitro transcription kit (Roche). This produces a single positive stranded RNA. The resulting RNA is transfected into BHK-21 cells in a 60 mm culture dish with DMEM supplemented with 2 % FBS using lipofectin reagent (Gibco BRL) as described by the manufacturer's protocol. The transfected cells are incubated overnight at 33 °C. Following the incubation, the medium is removed from the cells, replaced with DMEM containing 2 % serum, and incubated for 2–3 additional days at 33 °C until the cells began to lyse,

indicating virus production. Virus is isolated from the cells following the procedure described above (Subheading 3.2.1). The virus is amplified beginning with very small volumes until the virus titer reaches 10^4 PFU/ml, and then larger volumes can be used to produce the recombinant virus for infecting stocks. The viral titer of the recombinant viruses is determined by plaque assay as described above (Subheading 3.2.3).

3.2.5. Induction of TMEV-Induced Demyelinating Disease—TMEV is a naturally endemic infection in mice spread through the fecal oral route, which results in a 50–60 % incidence of TMEV-IDD in disease-susceptible animals. Experimentally, disease-susceptible SJL/J mice can be infected intracranially in order to increase the incidence of TMEV-IDD development to approximately 90 % of all infected animals. Six- to seven-week-old female SJL/J mice are anesthetized with aerosolized isoflurane (Abbott Laboratories) and inoculated with either 5×10^6 PFU of wild-type TMEV (BeAn 8386 strain) infecting stock (produced as described in Subheading 3.2.1) or recombinant mimic-expressing viruses (produced as described in Subheading 3.2.4), in 30 μ l in the right cerebral hemisphere by freehand injection with a 24 gauge needle. The cap of the needle remains on the needle during injection but is cut down so that approximately 2–3 mm of the needle is exposed for the injection. This same needle guard is used in all injections so that the virus is injected at the same depth in each mouse injected. Mice are marked to allow for individual evaluation of clinical and histological disease.

3.2.6. Clinical Assessment of TMEV-Induced Demyelinating Disease—The clinical disease presentation seen in susceptible mouse strains, such as SJL/J, depends upon the strain of TMEV used for infection. Following inoculation with the brain-derived DA strain of virus, mice first develop a flaccid paralysis. Mice recover in approximately 2 weeks indicating that this phase of disease is self-limiting. However, 2–3 weeks after infection, mice then develop a spastic paresis of the hind limbs, which, in SJL/J mice, has a chronic disease course resulting in severe spastic paralysis (42). In contrast, infection of SJL/J mice with the tissue culture-adapted BeAn 8386 strain does not produce clinical evidence of a first-phase disease. Infected mice begin to show signs of clinical disease between 30 and 40 days post-TMEV infection and develop a chronic, progressive paralysis with no recovery or remitting episodes, similar to primary progressive MS. Unlike EAE, clinical signs develop slowly, with no drastic changes in gait from day to day. Mice are monitored for disease progression 2–3 times per week continuing for 100 days post-infection. Each mouse is assigned a numerical score between 0 and 5, based on the severity of its impairment: 0, asymptomatic; 1, mild gait abnormalities; 2, severe gait abnormalities; 3, loss of ability to right itself associated with mild spastic paralysis; 4, spastic paralysis in both hind limbs combined with urinary incontinence and dehydration; 5, moribund. Infection with recombinant infection with recombinant TMEV containing different molecular mimic sequences leads to different disease profiles depending upon the epitope expressed. Mice infected with the leader deletion recombinant virus, Δ Clal-BeAn, do not develop signs of demyelinating autoimmune disease (48). In contrast, mice infected with the recombinant PLP139-BeAn virus exhibit an earlier onset and more severe clinical disease, with onset between days 7 and 10 post-infection (48).

3.2.7. Immunological Aspects of TMEV-Induced Demyelinating Disease—A number of immunological assays can be performed to determine the specificity, class, and timing of the immune system in disease pathology following TMEV infection. Support for a $CD4^+$ T cell-mediated pathogenesis of TMEV-IDD derives from studies showing that susceptibility strongly correlates with the development of chronic, high levels of TMEV- and myelin-specific delayed-type hypersensitivity (DTH) reactions. DTH to TMEV capsid epitopes in BeAn-infected, susceptible SJL/J mice develops within 5–10 days post-infection,

preceding the appearance of clinical signs, and remains at high levels for at least 100 days post-infection (49). Previous data indicated that VP2_{74–86} was the immunodominant Th1 determinant in TMEV-infected SJL/J mice, as 80–90 % of the DTH response is directed against this virion peptide within the VP2 protein (50). However, recent work by Jin et al. shows a new immunodominant peptide within the 3D viral polymerase protein (3D_{21–36}) which a greater number of CD4⁺ T cells respond to compared to VP2_{74–86} (51). The immunodominant myelin epitope in SJL/J mice is PLP_{139–151} and responses to this self-antigen are first observed around 45–50 days post-TMEV infection, i.e., 2–3 weeks after clinical disease onset (37). As disease progresses, epitope spreading occurs in a hierarchical order with intermolecular spreading to PLP_{178–191} and PLP_{56–70} and intramolecular epitope spreading to MOG_{92–106} and MBP_{84–104}, occurring during the chronic late phase of TMEV-IDD, by day 100 post-infection (36, 37). These responses can be detected by both DTH and splenic T cell proliferative responses.

The early detection of PLP_{139–151} responses, between days 10 and 14 post-infection, can be observed following the infection of mice with PLP139-BeAn (48). This is in contrast to wild-type TMEV-infected mice where myelin responses arise by day 50 post-infection. In addition, there is evidence for epitope spreading of myelin epitopes to PLP_{178–191} in PLP139-BeAn-infected mice (48, 52).

In addition, cytokines play an important role in TMEV-IDD. The release of the pro-inflammatory cytokines (IFN- γ and LT/TNF β) by both viral and myelin-specific Th1 cells in the CNS leads to the recruitment and activation of monocytes and macrophages which cause myelin destruction by a terminal nonspecific *bystander* mechanism (36). These cytokines can be quantitated using ELISA, ELISPOT, and other cytokine measures.

In conclusion, following TMEV infection of the CNS, bystander damage to myelin is initiated by virus-specific CD4⁺ Th1 cells, which leads to the release, processing, and presentation of myelin auto-antigens by CNS APCs. This presentation to autoreactive T cells leads, via epitope spreading, to an autoimmune response directed against CNS myelin which perpetuates chronic clinical pathology.

4. Notes

1. These protocols have been developed by numerous laboratories, with some variation between each protocol. Variations in optimal culture conditions and immunization conditions are apparent. As such, it is important for each laboratory to optimize the culture system for its environment and reagents. Thus, listed concentrations are only approximations.
2. Susceptible strains: Varieties of mouse strains are used to study EAE. The most common are SJL/J, B10.PL, C57BL/6, C3H, SWR, and the F₁ progeny of several of these parental strains. The importance of pertussis toxin and the type of disease course (chronic versus relapsing/remitting) for each of these strains is discussed below. Table 1 describes the haplotype and reported encephalitogenic peptides for each of these susceptible strains.

Resistant strains: While many mouse strains are useful in the study of EAE, not all mouse strains appear to be susceptible to EAE induction. For instance, A/J, C3H/HeJ, AKR, NZW, and DBA/2 appear to be resistant to EAE after priming with known myelin antigens (53).

3. For efficient disease induction in some strains, administration of pertussis toxin is required on days surrounding peptide priming. 200 ng (200 μ l of a 1 μ g/ml stock in PBS) should be administered intraperitoneally on the day of priming and again 2

days later. Pertussis should be dissolved at least 24 h prior to use to prevent death associated with administration of fresh pertussis. Pertussis toxin is required to initiate EAE in C57BL/6, B10.PL, and their F₁ progeny, as well as (SJL/J × BALB/c)F₁ mouse strains. Efficient disease induction in SJL/J mice requires the use of pertussis toxin only with certain neuroantigens, e.g., intact MBP and MPB_{84–104}.

4. In addition to induction of disease and clinical disease, several other techniques have been used to assay the presence and persistence of T cell responses (and importantly antigen-specific responses) following the development of EAE. These include DTH (54), proliferation assays (55), immunohistochemistry (56), and histopathology (Fig. 1). Complete descriptions of these techniques can be found in the respective citations.
5. The disease course and immune reactivity described in these methods relate to the BeAn strain of TMEV. The DA strain also induces a demyelinating disease with some differences in clinical disease and different immune reactivity. In addition, the GDVII strain of TMEV induces a lethal encephalitis and thus does not result in a late-onset demyelinating disease.

The mouse strain described in these methods is the SJL/J mouse which is susceptible to BeAn strain TMEV-IDD. C57BL/6 are resistant to BeAn strain TMEV-IDD. BALB/c have varying susceptibility to TMEV-IDD depending on the substrain, where BALB/cAnNCr are mildly susceptible, and the BALB/cByJ are resistant to TMEV-IDD. Additionally, the C57BL/6 × SJL/J F₁ strain of mice has an intermediate frequency of TMEV-IDD development with approximately 30 % of intracranially infected animals developing mild to moderate disease symptoms.

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Fig. 1. Histopathologic evaluation of 1 μm thick Epon embedded spinal cord sections. Panel (a)—spinal cord section from a normal mouse. Note the presence of profuse and evenly distributed ringed structures reflecting myelinated axons, with no infiltrating immune cells. Panel (b)—spinal cord section from an SJL/J mouse with severe EAE. Note the few and unevenly distributed myelinated axonal ringed structures with large bare areas, along with large numbers of infiltrating immune cells throughout the section, appearing as dense and dark spots. Magnification: $\times 220$.

Table 1

Mouse strain and encephalitogenic peptides in active EAE

Mouse strain	H-2 type	Peptide ^a	Sequence ^a	Reference
SJL/J	H-2 ^s	MBP _{89–101}	VHFFKNIVTPRTP	(57)
		MBP _{84–104}	VHFFKNIVTPRTPPSQKGR	(4)
		PLP _{139–151} ^b	HSLGKWLGHDPKF	(55, 58)
		PLP _{104–117}	KTTCGKGLSATVT	(58)
		PLP _{178–191}	NTWTTCQSIAPPSK	(59)
		PLP _{57–70}	YEYLINVIHAFQYV	(60, 61)
		MOG _{92–106}	DEGGYTCFFRDHSYQ	
PL/J, B10.PL	H-2 ^u	MBP _{Ac1–11}	Ac-ASQKRPQRHG	(62)
		PLP _{178–191}	NTWTTCQSIAPPSK	Unpublished (B10.PL)
		MBP _{35–47}	TGILDSIGRFFSG	(62)
		PLP _{43–64}	EKLIETYFSKNYQDYEYLINVI	(63)
(PL/J × SJL/J)	H-2 ^{s/u}	MBP _{Ac1–11}	Ac-ASQKRPQRHG	(62)
F1		PLP _{43–64}	EKLIETYFSKNYQDYEYLINVI	(63)
		PLP _{139–151}	HSLGKWLGHDPKF	(55)
C57BL/6	H-2 ^b	MOG _{35–55}	MEVGWYRSPFSRVVHLYRNGK	(64)
		PLP _{178–191}	NTWTTCQSIAPPSK	(65)
C3H	H-2 ^k	PLP _{103–116}	YKTTICGKGLSATV	(66)
SWR	H-2 ^q	PLP _{215–232}	PGKVCGSNLLSICKTAEF	(67)
(SJL/J × B10.PL)	H-2 ^{s/q}	PLP _{139–151}	HSLGKWLGHDPKF	Unpublished
		PLP _{178–191}	NTWTTCQSIAPPSK	Unpublished
		MBP _{Ac1–11}	Ac-ASQKRPQRHG	Unpublished
(SJL/J × C3H/HeJ)F1 ^c	H-2 ^{s/k}	PLP _{190–209}	SKTSASIGSLCADARMYGVL	(68)
		PLP _{215–232}	PGKVCGSNLLSICKTAEFQ	(60)
BALB/cPt ^c	H-2 ^d	PLP _{178–191}	NTWTTCQSIAPPSK	(59)
NOD	H-2 ^{g7}	PLP _{48–70}	TYFSKNYQDYEYLINVIHAFQYV	(69)
		MOG _{35–55}	MEVGWYRSPFSRVVHLYRNGK	(70)

^aSequences for MBP peptides are based on different species variants of MBP, which have different numbering systems; sequences for PLP peptides are based on the mouse sequence. The reader is urged to consult the indicated references for more detailed information

^bThe PLP_{139–151} sequence has a serine (S) for cysteine (C) substitution at position 140 to enhance solubility

^cThe EAE observed in these mice is nonclassical. In (SJL/J × C3H/HeJ)F₁ mice, the disease causes imbalance and axial rotatory movement (rotatory EAE). In BALB/cPt, mice show lack of balance and forelimb paralysis in the absence of hindlimb paralysis

Table 2Summary of general parameters for various EAE adoptive transfer models ^a

Mouse strain	Antigen specificity	Donor immunization period (days)	In vitro antigen concentration (µg/ml)	In vitro IL-12 (ng/ml)	In vitro culture time (h)	Number of blast transfers (×10 ⁶)	Disease type	Disease severity
SJL/J	PLP	7–14	50–100	–	72–96	5–10	Relapsing–remitting	Severe
(27, 71–78)	PLP _{139–151}	7–14	20	–	72–96	1–5	Relapsing–remitting	Severe
	MBP	7–14	50–100	–	72–96	40–60	Relapsing–remitting	Moderate
	MBP _{84–104}	7–14	50	–	72–96	10–20	Relapsing–remitting	Moderate
C57BL/6	MBP	10–14	50–100	–	72–96	50	Monophasic/chronic	Mild
(64, 79–82)	MBP _{84–104}	10–14	50	–	72–96	50	Monophasic/chronic	Mild
	MOG	10–14	50	25	72–96	20	Relapsing–remitting	Moderate
	MOG _{35–55}	10–14	10	25	72–96	20	Relapsing–remitting	Moderate
B10.PL ^b (43)	MBP _{Ac1–11}	–	50	10	72	1	Monophasic	Moderate
B10.S (43)	MBP	10–11	25	20	96	35	Monophasic	Moderate
	MBP _{87–106}	10–11	50	20	96	35	Monophasic	Moderate

^aThese parameters have been optimized in different laboratories as previously described. *SJL/J protocol parameters from refs. 27, 71–78, C57BL/6 protocol parameters from refs. 64, 79–82, B10.PL protocol parameters from ref. 43, and B10.S protocol parameters from ref. 43

^bNote that the B10.PL system employs T cell receptor transgenic donors which do not require in vivo priming, only in vitro culture with MBP_{Ac1–11} peptide and rIL-12