Video Article

A Simple Approach to Induce Experimental Autoimmune Neuritis in C57BL/6 Mice for Functional and Neuropathological Assessments

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Abstract

Experimental autoimmune neuritis (EAN) is a well-appreciated experimental model of autoimmune peripheral demyelinating diseases. EAN disease is induced by immunizing mice with neurogenic peptides to direct an inflammatory attack toward components of the peripheral nervous system (PNS). Recent advances have enabled the induction of EAN in the relatively resistant C57BL/6 mouse line using myelin protein zero (P0)₁₀₆₋₁₂₅ or P0₁₈₀₋₁₉₉ peptides delivered in adjuvant combined with the injection of pertussis toxin. The ability to induce EAN in the C57BL/6 strain allows for the use of the numerous genetic tools that exist on this mouse background, and thus allows the sophisticated study of disease pathogenesis and interrogation of the mechanistic action of novel therapeutics in combination with transgenic approaches. In this study, we demonstrate a simple approach to successfully induce EAN using the P0₁₈₀₋₁₉₉ peptide in C57BL/6 mice. We also outline a protocol for the assessment of functional deficits that occur in this model, accompanied by an array of neuropathological features. Thus, this model is a powerful experimental model to study the pathogenesis of human peripheral demyelinating neuropathies, and to determine the efficacy of potential therapies that aim to promote myelin repair and protect against nerve damage in autoimmune neuritis.

Video Link

The video component of this article can be found at <https://www.jove.com/video/56455/>

Introduction

Peripheral neuropathies can be either genetic in origin or acquired, with acquired neuropathies having either metabolic, ischaemic, inflammatory, or toxic precipitants. These diseases are also usefully classified as either axonal or demyelinating in origin. The most common acquired demyelinating peripheral neuropathies are Acute Inflammatory Demyelinating Polyneuropathy (AIDP, also known as Guillain-Barré syndrome, GBS) and Chronic Inflammatory Demyelinating Polyneuropathy (CIDP)^{1,2,3,4}; both are pathogenetically characterized by an autoimmune reaction directed against the myelin sheath, causing demyelination of the peripheral nerves. In these diseases, activated T cells cross the blood nerve barrier and generate an immune reaction within the PNS. Activation of macrophages within the nerve then causes demyelination either directly via phagocytic attack or indirectly via secreted inflammatory mediators, resulting in clinical disabilities such as paralysis and sensory dysfunction⁵. While demyelinated axons retain the ability to be remyelinated following demyelination, remyelination is often delayed or incomplete, resulting in susceptibility of the naked axons to irreversible damage, which is the major cause of permanent clinical disability. Currently, the most effective treatments are immunomodulatory, but despite their efficacy, in many cases the recovery is often slow and ~25% patients will experience residual functional deficits that significantly reduce their quality of life^{6,7}.

EAN is a widely used animal model of demyelinating peripheral neuropathy that has provided valuable insights into pathogenesis and a means to assess novel therapeutic agents⁴. This model can be induced in different species such as rabbits, rats, mice, and guinea pigs, and is induced by immunization with neurogenic antigens. However, ultimately the successful EAN induction depends on an appropriate immune response for disease to occur. Given the species (and inter-species/strain) variations in immune function, multiple combinations of antigens and adjuvants have been developed to successfully induce EAN. In terms of murine genetic tools, the C57BL/6 is the most widely used; however, the traditional P2 protein peptide 57-81 (P2₅₇₋₈₁) that results in disease in the susceptible SJL mouse strain⁸ is unable to illicit pathogenesis leading to functional deficits in the C57BL/6 strain. Fortunately, sensitization paradigms using the P0₁₀₆₋₁₂₅ or P0₁₈₀₋₁₉₉ peptides, delivered in adjuvant combined with the injection of pertussis toxin can overcome this barrier, enabling sophisticated genetic tools to be utilized in the murine EAN model.

Here, a simple method for the induction of EAN in the C57BL/6 mice is presented. In addition, a comprehensive detailed approach by which to evaluate the functional and neuropathological deficits associated with the disease is provided. The P0₁₈₀₋₁₉₉ peptide⁹ was chosen in preference to the P0₅₇₋₈₁ alternative¹⁰. The P0₁₈₀₋₁₉₉ model has been described to produce less severe clinical signs compared with the P0₅₇₋₈₁ alternative¹⁰, and is therefore likely to withstand the introduction of potentially deleterious genetic perturbations, recover from surgical procedures (such

as osmotic pump implantation), and is amenable to treadmill gait function testing⁴. However, the treadmill gait function tests and histological protocols described here could easily be applied when studying the disease in a P0₅₇₋₈₁ induced variant.

Protocol

All procedures described here were approved by the Florey Institute for Neuroscience and Mental Health (Melbourne Brain Centre) Animal Ethics Committee and follow the Australian Code of Practice for the Use of Animals for Scientific Purposes.

1. EAN Induction

NOTE: EAN can be successfully induced in male C57BL/6 mice aged between 6-8 weeks. The induction protocol takes 9 days in total. Day 0 refers to the day of the first immunization. For this protocol, injections were conducted under anesthesia (see step 1.1.2 below).

1. On Day -1:

1. Prepare the Pertussis Toxin (see **Table of Materials**) solution of 1.6 µg/mL using the sterile 0.1 M mouse-isotonic phosphate buffered saline (MT-PBS).
2. Anesthetization with isoflurane:
 1. Place the mouse (C57BL/6, male, 6-8 weeks) in the anesthetization chamber and adjust the oxygen flow to 1 L/min.
 2. Turn on the isoflurane vaporizer, adjust it to 2.5% for anesthetization, and monitor breathing and wait for 2 min, or until primary reflexes (corneal and hind limb) are no longer responsive
3. Remove the mouse from the anesthetization chamber and administer 250 µL of the above Pertussis Toxin solution via an intraperitoneal (i.p.) injection using a 0.5 mL syringe with a 30^{1/2} G needle.
4. Prepare the injectable inoculum for immunization:
 1. Prepare **Solution A** using 2 mg/mL solution of P0₁₈₀₋₁₉₉ peptide (with > 98% purity, sequence S-S-K-R-G-R- Q-T-P-V-L-Y-A-M-L-D-H-S-R-S) in 0.9% saline.
 2. Prepare **Solution B** using 20 mg/mL solution of *Mycobacterium tuberculosis* (see **Table of Materials**) in Freund's complete adjuvant (FCA, comprising: 15% mannide monooleate + 85% of paraffin oil and 0.5 mg/mL of desiccated killed and dried *Mycobacterium butyricum*).
 3. To make the final inoculum for injecting, combine equal volume parts of Solutions A and B in a bead beater and mix them at a maximum speed for 1 min at room temperature.
NOTE: Solutions A and B can be kept as stock and stored at 4 °C for a maximum of one month but the combined final inoculum must be freshly prepared on the day of the mouse injection.

2. On Day 0, administer 50 µL of the inoculum (preparation outlined in step 1.1.4) into a mouse via a subcutaneous injection using a 23 G needle.
NOTE: The injection can be placed between the shoulder blades, or between the hind limb and tail over the caudal dorsum.
3. On Day 1, make the Pertussis Toxin solution of 1.2 µg/mL (in MT-PBS) and administer 250 µL into a mouse via i.p. injection using a 0.5 mL syringe with a 30^{1/2} G needle.
4. On Day 3, repeat step 1.3 above.
NOTE: Stock solutions A and B can be made up and stored at 4 °C for a maximum of one month. However, the final injectable inoculum, produced by combining stock solutions A and B, must be done on the day of the injection.
5. On Day 8, administer 50 µL of the inoculum (preparation outlined in step 1.1.4) into a mouse via a subcutaneous injection (see step 1.2 above), at the same injection site as in step 1.2 (for each animal)

2. Clinical Scoring

1. Perform clinical scoring out daily starting on Day 0.
2. Give each mouse a score of 0, 1, 2, 3, or 4 according to the published criteria⁴. See **Table 1** for EAN clinical scoring.
NOTE: For consistency, an effort should be made to score mice at the same time daily by the same researcher.

3. Motor Function Assessment

NOTE: The motor performance is assessed in parallel with clinical scoring for the same cohort of animals. The motor function assessment apparatus must be connected to a computer that has a gait function imaging system (see **Table of Materials**) installed. It is also recommended that all mice to be assessed should be habituated to the running task prior to EAN induction. To do this, practice runs (2 test runs per mouse) are performed three days prior to disease induction (Day -3).

1. Turn on the motor function assessment apparatus and switch on the light button.
2. Scruff the mouse firmly and ink its feet by lowering it onto a container filled with red ink while holding its tail.
NOTE: This step is required for the C57BL/6 strain, but can be skipped if a mouse strain with a white coat color is being used.
3. Place the mouse into the walking compartment and set the treadmill speed at 15 cm/s.
4. Turn on the treadmill and click the "record" button to capture the running motion of the mouse using the gait function imaging system (see **Table of Materials**).
5. Use a timer and measure each running for 36 s. After 36 s, stop recording and stop the treadmill.
NOTE: Mice that cannot successfully complete the running task for this 36 s interval are considered to have failed.
6. Save the video file in the designated folder.

7. Repeat the above steps for each mouse to be assessed. Test the mice with the same running task every 3 days.
8. Analyze the edited video files using software for gait function parameters (see **Table of Materials**).
NOTE: The details of how to analyze different motor parameters vary amongst software so please refer to the manufacturer's instruction before analysis. To gain histological evidence of axonal and myelin damage via either immunohistochemistry or electron microscopy, animal tissues can be taken at the any stage post motor function assessment depending on the specific aims of research.

Representative Results

P0₁₈₀₋₁₉₉ peptide induced EAN in C57BL/6 mice leads to a monophasic disease with clinical score onset from 6 days post first immunization (dpi), and maximal score severity is observed from 25 dpi followed by some clinical score improvement from 40 dpi duration (**Figure 1**)^{4,9}. In terms of gait function, mice begin to fail at a simple running task as early as 6 dpi, and by 35 dpi mice have little capacity to complete a simple treadmill running task and do not improve in running ability even at 55 dpi⁴ (**Figure 1**). Mice induced with EAN progressively declined in their ability to perform the running task over time. Running task failure is observed in 80% of mice, but in addition to this, hind limb gait widening in treadmill running is a key functional deficit that does not resolve over the duration of disease⁴.

There are several key neuropathological features in P0₁₈₀₋₁₉₉ induced EAN: damage to the myelin sheath; axon stress; and increased node width and damage to paranodal structures. Examination of semi-thin sections taken from sciatic nerves reveals areas of demyelination (**Figure 2B**, arrows) compared to sciatic nerves from age-matched healthy controls (**Figure 2A**). Importantly, it is possible to obtain G-ratios from images captured from semi-thin sciatic nerve sections imaged at high power with light microscopy⁴. However, high power transmission electron microscopy (TEM) images are required to identify specific neuropathological features such as dysmyelination, damage to the myelin lamella, and signs of axon stress such as mitochondrial swelling (disarrangement and distortion of cristae and partial or total cristolysis) (**Figure 2C-2D**). Consistent with the ultrastructural evidence of axon stress (**Figure 2C-2D**), the P0₁₈₀₋₁₉₉ induced EAN model also displays acute axonal damage, demonstrated by dramatically elevated β -amyloid precursor protein (APP) expression in the sciatic nerves (**Figure 3A**). This is not observed in the age-matched healthy control tissues (**Figure 3A**). Neuropathological changes at the nodes and the paranodes have been well documented in GBS; in particular, the AMAN variant^{11,12}. In our murine EAN model, we identify that the widening of the nodes and disruption to the paranode are additional neuropathological features of P0₁₈₀₋₁₉₉ induced EAN (**Figure 3B**).

Figure 1: Schematic representation of the EAN induction paradigm, clinical score assessment, and running capacity during EAN. (A) Overview of EAN induction protocol in C57BL/6 mice, commencing from 6-8 weeks of age, with schematics of (B) expected clinical scores and (C) running times during disease course (see reference⁴). [Please click here to view a larger version of this figure.](#)

Figure 2: Mice with EAN display myelin loss, myelin damage, and signs of axons stress. (A) A bright field image (under 100X magnification) taken from semi-thin sections of sciatic nerves from age-matched healthy control. (B) A bright field image (under 100X magnification) taken from semi-thin sections of sciatic nerves from mice induced with EAN at 55 days post first immunization (dpi). Areas lacking myelin surrounded by thinly myelinated axons (arrowheads) indicate remyelination; this was not observed in tissue obtained from aged-matched healthy controls (see A). (C) A representative electron microscopy image (under 5,000X magnification) taken from sciatic nerves from panel B demonstrating myelin pathology (open arrow) and axon stress (arrowheads indicating swollen mitochondria at 55 dpi). (D) A high power TEM image (bounding box in C) demonstrating swollen mitochondria that are indicative of axon stress (arrow heads), but this is absent from age-matched healthy controls (data not shown). Scale bars = 10 μ m (A, B), and 500 nm (C, D). [Please click here to view a larger version of this figure.](#)

Figure 3: Axon stress and nodal pathology is a feature of P0₁₈₀₋₁₉₉ induced EAN. (A) Single plane confocal images (under 40X magnification) taken through the sciatic nerves at 55 dpi. Tissue from EAN mice show dramatically elevated APP expression co-localized with β -III tubulin (a marker of neurons) indicating signs of axonal stress. (B) High power Z-projections (100X magnification) of paranodes stained with an antibody against Caspr in sciatic nerve sections. In nodes from EAN mice, there is an increased distance between adjacent paranodes indicating node widening. In addition, nodal pathology such as disruption to the paranode structure can be observed in EAN mice but is absent from age-matched healthy controls. Scale bars = 10 μ m (A), and 5 μ m (B). [Please click here to view a larger version of this figure.](#)

Score	Criteria	Notes and comments of assessment criteria
0	Normal mouse	
1	Less lively OR lathery	This is assessed in response to normal handling, mice typically first show signs of reduced activity at from Day 5 post immunization
2	Tail paresis OR	Tail paresis is assessed by the tail stroke test (flicking the tail).
	Mild limb paresis	Mild limb paresis refers to mice drag their belly on the ground when walking on a flat surface
3	Tail paresis + Mild limb paresis OR	Mild ataxia is assessed as any difficulty in normal walking
	Tail paresis + Mild ataxia OR	
	Mild limb paresis + Mild ataxia	
4	Severe ataxia	Severe ataxia is identified by atypical backwards walking, combined with either limb paresis, mild limb paresis, or tail paresis

Table 1: EAN clinical score paradigm.

Discussion

This report outlines a simple method to induce EAN using the P0₁₈₀₋₁₉₉ peptide in C57BL/6 mice, enabling the quantification of key neuropathological and functional deficits in mice induced with EAN. Distinct to the EAN induction protocol described here is the use of anesthesia while performing the immunization injections. The use of isoflurane anesthesia greatly enhances the ability to ensure that the total volume of inoculum is injected subcutaneously in the desired location with minimal error and stress to the animals.

Mice will begin to display clinical signs of disease from 6 dpi⁴ using this method of EAN induction. However, disease onset as measured by clinical score has previously been reported to occur from ~10 dpi^{9,10}. While this difference in disease onset as determined by clinical score could be partially due to different clinical scoring criteria, it is more likely explained by the use of different adjuvants in the inoculum for EAN induction. This protocol uses a Freund's complete adjuvant containing *M. butyricum*, which is supplemented with *M. tuberculosis*. Other studies use Freund's adjuvant supplemented only with *M. tuberculosis* in the injected inoculum containing the P0₁₈₀₋₁₉₉ peptide^{9,10} for immunization.

In this protocol, delivering pertussis toxin via i.p. injection is recommended in preference to intravenous (i.v.) tail vein injection. It has been shown that the mode of pertussis delivery does not significantly alter disease onset or progression⁴, but the choice to deliver via the i.p. route, as opposed to the i.v. tail vein, is motivated by risk mitigation and an attempt to reduce the chance of operating error. Frequently, tail vein i.v. injections are technically more challenging compared with injections of an equal volume. It has been previously shown that the use of pertussis toxin as an adjuvant is necessary to induce EAN when using the P0₁₈₀₋₁₉₉ peptide⁹. Interestingly, previous work has shown that halving the dose of pertussis outlined in this method, is sufficient to illicit EAN measured by clinical score, neuropathology, and gait widening during treadmill running⁴. However, decreasing the dose of pertussis toxin used in during EAN induction significantly influences running capacity, and mice perform significantly better at the treadmill running task when EAN is induced with half the pertussis dose described in this method⁴.

The motor deficits observed in this P0₁₈₀₋₁₉₉ peptide induced EAN model is accompanied and supported by strong neuropathological evidence. Previously, it has been demonstrated that quantifying the extent of myelin damage is sensitive enough to detect alterations in disease severity that resulted from halving the dose of pertussis toxin during EAN induction. Here, other hallmarks of EAN such as axonal stress and disruption to node/paranodal structures are identified, resulting in a suite of quantitative neuropathological markers for P0₁₈₀₋₁₉₉ induced EAN. This enables for more prudent examinations of neuropathology when evaluating disease processes, or the efficacy of novel therapeutic agents. Excitingly, these neuropathological assessments may be correlated to functional readouts in a bid to provide deeper insights and a better understanding of the relationship between neuropathology and the function deficits that are a feature of this model of EAN. In addition to the neuropathological analyses and gait function test employed in this study, other functional assessments such as nerve conduction velocity measurement are suggested in order to fully capture function outcomes of therapeutic interventions using this model.

EAN is a powerful and commonly used experimental model to examine peripheral demyelination and remyelination, with the sciatic nerves and cauda equina being the most frequently investigated peripheral nerves. The major advantage of EAN is that it results in a reproducible and quantifiable motor deficit, thus allowing analyses of neuroprotection and myelin repair from a clinical perspective. Further, the myelin damage is acute and reproducible thus allowing detailed analyses of myelin and axonal damage from a histopathological perspective.

While EAN model represents an exciting experimental mean to investigate demyelinating peripheral neuropathy, there are potential limitations as well as technical challenges. It is important to appreciate the heterogeneity nature of demyelinating peripheral neuropathy in humans, involving a complex interaction between the immune system and PNS. The current challenge is that no one EAN animal model can faithfully mimic all its aspects. Further, there are substantial inter-species and inter-strain variations regarding the disease severity and progression of the EAN model. For example, we found that overall the EAN induced in C67/B6 mice is less severe than the EAN induced in Lewis rats (unpublished data). Within the same strain, there is also gender difference. Our unpublished data show that the EAN model induced in male C57/B6 mice is more reproducible than the one induced in female mice, however, the exact reason underpinning this gender difference is unclear.

In conclusion, we reported a protocol that allows a robust and rapid induction of the EAN model in male C57/B6 mice. Combining clinical scoring and motor function assessment with neuropathological studies provides a strategic tool for studying demyelinating peripheral neuropathy from both clinical and pathological perspectives. Importantly, it promotes future studies on the mechanisms of therapies that target peripheral nerve myelin repair using transgenic mouse approaches.

Disclosures

The authors have no conflicts of interest regarding this work.

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References

1. Newswanger, D. L., Warren, C.R. Guillain-Barre syndrome. *Am Fam Physician*. **69** (10), 2405-2410 (2004).
2. Ruiz, E., Ramalle-Gomara, E., Quinones, C., Martinez-Ochoa, E. Trends in Guillain-Barre syndrome mortality in Spain from 1999 to 2013. *Int J Neurosci*. **126** (11), 985-988 (2016).
3. Walling, A. D., Dickson, G. Guillain-Barre syndrome. *Am Fam Physician*. **87** (3), 191-197 (2013).
4. Gonsalvez, D. G. *et al.* A Functional and Neuropathological Testing Paradigm Reveals New Disability-Based Parameters and Histological Features for P0180-199-Induced Experimental Autoimmune Neuritis in C57BL/6 Mice. *J Neuropathol Exp Neurol*. **76** (2), 89-100 (2017).
5. Berg, B. *et al.* Guillain-Barre syndrome: pathogenesis, diagnosis, treatment and prognosis. *Nat Rev Neurol*. **10** (8), 469-482 (2014).
6. Koller, H., Schroeter, M., Kieseier, B. C., Hartung, H. P. Chronic inflammatory demyelinating polyneuropathy—update on pathogenesis, diagnostic criteria and therapy. *Curr Opin Neurol*. **18** (3), 273-278 (2005).
7. Griffin, J. W. *et al.* Pathology of the motor-sensory axonal Guillain-Barre syndrome. *Ann Neurol*. **39** (1), 17-28 (1996).
8. Taylor, W. A., Hughes, R. A. Experimental allergic neuritis induced in SJL mice by bovine P2. *J Neuroimmunol*. **8** (2-3), 153-157 (1985).
9. Zou, L. P. *et al.* P0 protein peptide 180-199 together with pertussis toxin induces experimental autoimmune neuritis in resistant C57BL/6 mice. *J Neurosci Res*. **62** (5), 717-721 (2000).
10. Miletic, H. *et al.* P0(106-125) is a neuritogenic epitope of the peripheral myelin protein P0 and induces autoimmune neuritis in C57BL/6 mice. *J Neuropathol Exp Neurol*. **64** (1), 66-73 (2005).
11. Hafer-Macko, C. *et al.* Acute motor axonal neuropathy: an antibody-mediated attack on axolemma. *Ann Neurol*. **40** (4), 635-644 (1996).
12. Griffin, J.W. *et al.* Early nodal changes in the acute motor axonal neuropathy pattern of the Guillain-Barre syndrome. *J Neurocytol*. **25** (1), 33-51 (1996).