Video Article

Myelin Oligodendrocyte Glycoprotein (MOG_{35-55}) Induced Experimental Autoimmune Encephalomyelitis (EAE) in C57BL/6 Mice

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Abstract

Multiple sclerosis is a chronic neuroinflammatory demyelinating disorder of the central nervous system with a strong neurodegenerative component. While the exact etiology of the disease is yet unclear, autoreactive T lymphocytes are thought to play a central role in its pathophysiology. MS therapy is only partially effective so far and research efforts continue to expand our knowledge on the pathophysiology of the disease and to develop novel treatment strategies. Experimental autoimmune encephalomyelitis (EAE) is the most common animal model for MS sharing many clinical and pathophysiological features. There is a broad diversity of EAE models which reflect different clinical, immunological and histological aspects of human MS. Actively-induced EAE in mice is the easiest inducible model with robust and replicable results. It is especially suited for investigating the effects of drugs or of particular genes by using transgenic mice challenged by autoimmune neuroinflammation. Therefore, mice are immunized with CNS homogenates or peptides of myelin proteins. Due to the low immunogenic potential of these peptides, strong adjuvants are used. EAE susceptibility and phenotype depends on the chosen antigen and rodent strain. C57BL/6 mice are the commonly used strain for transgenic mouse construction and respond among others to myelin oligodendrocyte glycoprotein (MOG). The immunogenic epitope MOG_{35-55} is suspended in complete Freund's adjuvant (CFA) prior to immunization and pertussis toxin is applied on the day of immunization and two days later. Mice develop a "classic" self-limited monophasic EAE with ascending flaccid paralysis within 9-14 days after immunization. Mice are evaluated daily using a clinical scoring system for 25-50 days. Special considerations for care taking of animals with EAE as well as potential applications and limitations of this model are discussed.

Video Link

The video component of this article can be found at https://www.jove.com/video/51275/

Introduction

Multiple sclerosis (MS) is a chronic demyelinating inflammatory disease of the central nervous system wherein the destruction of oligodendrocytes and neurons results in heterogenic and accumulating clinical symptoms. MS is considered as a prototypic autoimmune disorder of the central nervous system (CNS) and animal models have been developed to shed light on its complex pathogenesis. Furthermore, current therapies are only partially effective and target mainly the inflammatory phase of the disease while the neurodegenerative component is probably the major challenge for future therapeutic approaches[1,2].

While the exact etiology of the disease is yet unclear, an autoimmune reaction against epitopes on the myelin sheath of the axons in the CNS is assumed to provoke the onset of the disease. Dysregulation of the immune system, genetic vulnerability and environmental factors (*e.g.* infections, Vitamin D) are believed to influence central aspects of the pathophysiological mechanisms of MS.

Three different types of animal models are currently established for the exploration of pathologic patterns of MS: Viral models like Theiler's murine encephalomyelitis virus (TMEV), models induced by toxic agents like cuprizone, and finally different variants of experimental autoimmune encephalomyelitis (EAE)[3,4]. Although they all mimic features of MS, they differ tremendously in underlying pathological features like the involvement of the adaptive immune system. EAE is the most common animal model as it is especially useful to investigate neuroinflammatory pathways and often serves as a "proof-of-principle" model for the efficacy of novel treatment strategies[5,6]. EAE can be induced in many different animals (*e.g.* mice, rats, miniswine, guinea pigs, chickens, or primates). However, mice have become the most widely use species which is at least partly due to expanding repertoire of sophisticated transgenic or knockout mice[7].

The pathophysiology of EAE is based on the reaction of the immune system against brain-specific antigens. This reaction induces inflammation and destruction of the antigen carrying structures, resulting in neurological and pathological features comparable which those observed in MS patients. Three different approaches can be distinguished: Actively-induced EAE (aEAE; active immunization), passively transferred EAE (pEAE; transfer of encephalitogenic cells from an immunized animal), and more recently spontaneous EAE mouse models (sEAE) which allow the study

of autoimmune mechanisms without exogenous manipulation. The easiest inducible model is aEAE in mice yielding in fast and robust results. This model is considered as the "gold standard" of neuroimmunological animal models by many researchers in the field[8].

For aEAE induction, the animal is immunized with a subcutaneous injection of an emulsion consisting of the chosen antigen and complete Freund's adjuvans (CFA) accompanied by an intraperitoneal injection of pertussis toxin on the day of immunization and two days later. Consequently, myelin-specific T lymphocytes are activated in the periphery and migrate into the CNS across the blood-brain-barrier. Upon entry into the CNS, T cells are reactivated by local and infiltrating antigen-presenting cells resulting in subsequent inflammatory cascades, involvement of other cells like monocytes or macrophages and eventually in demyelination and axonal cell death[9]. Depending upon the immunization protocol and combination of mouse strain (*e.g.* C57BL/6, SJL/J, Biozzi) and antigen (*e.g.* myelin oligodendrocyte glycoprotein (MOG), myelin basic protein (MBP), myelin proteolipid protein (PLP)), the disease course can take an acute, chronic progressive or relapsing remitting disease course.

C57BL/6 mice have become the most commonly used strain for transgenic mice construction and a growing multitude of knockout or transgenic mice is available. We here describe a protocol for immunization of C57BL/6 mice with MOG_{35-55} peptide[10] which results in a monophasic EAE with first symptoms after 9-14 days, disease maximum about 3-5 days after disease onset and slow and partial symptom recovery over the next 10-20 days. As the immunogenic potential of MOG_{35-55} peptide alone is not sufficient to induce disease, adjuvants such as CFA are necessary. It is assumed, that the components of CFA activate mononuclear phagocytes inducing the phagocytosis of these molecules and the secretion of cytokines. This results in the prolongation of the presence of antigens and a more efficient transport of these to the lymphatic system. EAE induction is facilitated by application of pertussis toxin (PT) which has among others been suggested to modulate the blood-brain barrier and the immunological responsiveness itself[11]. After disease induction, special care must be taken for daily evaluation of mice for disease symptoms.

Protocol

1. General Comments for Mouse Experiments

1. All experiments using mice should be performed in accordance with the guidelines of the respective institutional animal care and use committee.
2. Keep the mice under pathogen-free conditions and enable them to have access to food and water *ad libitum*. Note: It is important to use age- and sex-matched mice in experimental groups because susceptibility to disease can vary with age and gender.
3. Depending on the chosen experimental conditions, a sham-immunized control group can be considered where MOG_{35-55} peptide is replaced by either PBS without antigen or a nonencephalitogenic peptide.
4. Please consider methodological aspects prior to starting experiments (see also below). We recommend to involve one or two blinded observers for EAE scoring.

2. Preparation of MOG_{35-55} Emulsion

1. 200 µl of a 1:1 ratio of MOG_{35-55} peptide solution and CFA should be injected to each mouse. There is some loss of the viscous emulsion while preparing and injecting. Therefore, prepare 1.5-2x of the needed amount. Calculate the total volume of the emulsion and divide by 2 for the needed amounts of both MOG_{35-55} peptide solution and CFA.
2. Dilute lyophilized MOG_{35-55} in ddH_2O to a final concentration of 2 mg/ml. We usually use 200 µg MOG_{35-55} peptide per mouse. This amount is contained in 100 µl of the stock solution. The peptide solution should be stored at -20 °C.
3. Place the content of 1 vial of desiccated Mycobacterium tuberculosis H37RA (100 mg) into a mortar.
    1. Ground with mortar and pestle to obtain a thin powder.
    2. Add 10 ml of incomplete Freund's adjuvants to obtain a 10 mg/ml CFA stock solution which can be stored at 4 °C.
    3. Prior to immunization, dilute CFA stock solution with IFA to a final concentration of 2 mg/ml. Mix thoroughly before each use to resuspend particulate material and consider some volume loss of the viscous solution during the experimental preparations.
    4. Mix 1:1 with MOG_{35-55} peptide solution until the final concentration of 1 mg/ml is reached.
    CAUTION: Heat-killed Mycobacterium tuberculosis stimulates the innate immune response. Avoid inhalation, ingestion and contact with skin and eyes.
4. Precool solutions on ice.
    1. Draw up 1 ml of 2 mg/ml CFA and 1 ml of 2 mg/ml MOG_{35-55} solution into two 2 ml syringes. Calculate the number of syringes necessary according to the number of immunized animals.
    CAUTION: Strictly avoid stitching during preparation of adjuvant as it may cause granulomas or induce autoimmune reactions.
    2. Use a 27 G cannula for the MOG_{35-55} solution and a 20 G cannula for CFA. Avoid air bubbles and connect both syringes with a three-way-valve.
    3. Send the emulsion from one syringe to the other and mix thoroughly for at least 10 min as a good emulsification is a critical step. Near-close the three-way-valve to support emulsification. The solution should be white, stiff and viscous with no separation of phases.
    4. Emulsion can be stored for several days prior to immunization. Wait at least 30 min after preparing the emulsions to observe whether they are stable. Prior to immunization, draw the solution into one of two syringes and connect a 27 G cannula.

3. Preparation of Pertussis Toxin

1. Different amounts of pertussis toxin can be found in the literature which also depends on the route of administration (*e.g.* intravenously or intraperitoneally). Our laboratory uses 400 ng of pertussis toxin in 200 µl of PBS intraperitoneally at the day of immunization and two days later. CAUTION: Pertussis toxin has many biological effects. Avoid inhalation, ingestion, and contact with skin and eyes.
2. Reconstitute 50 µg of pertussis toxin in 500 µl ddH_2O for a 100 µg/ml stock solution. Store at 4 °C.

3. Dilute 1:50 with PBS. 200 µl now contain 400 ng of PBS. Prepare the necessary amount of syringes and consider an additional excess volume of ~100 µl for each needle hub.

4. Animal Immunization

1. Please refer to the institutional animal care and use committee for specific criteria for required short-term euthanasia. Our laboratory uses brief narcotization with isoflurane while other protocols such as ketamine/xylazine injection or halothan may also be possible. Wait for anesthesia and use a front foot toe pinch to assess the level of anesthesia.
2. Make sure that immunization is performed by an experienced person to minimize stress for animals and to ensure optimal immunization.
3. Inject 100 µl of antigen/CFA emulsion subcutaneously into two different sites on each hind flank. Ensure that a bulbous mass forms under the skin which should persist throughout the experiment.
4. Inject 200 µl of pertussis toxin intraperitonelly.
5. Ensure that individual mice can easily be identified for daily evaluation, *e.g.* by color markings on the tail base.
6. Administer a second dose of pertussis toxin on day two after immunization.

5. EAE Monitoring

1. Weight and clinical score should be evaluated daily. The onset of EAE typically correlates with weight loss, which can be used as an indicator of disease activity. Please refer to the institutional animal care and use committee to predefine criteria when individual mice have to be taken out of the experiments. This should be considered when weight loss exceeds 20% of the initial body weight or when severe clinical signs (EAE score 7 or worse) occur. Please refer to the respective guidelines of the respective institutional animal care and use committee for allowed maximum scores.
2. When mice have clinical symptoms of EAE, it is important to ensure that the water bottle can still be reached and that food is placed on the cage floor.
3. Different scoring symptoms can be used. In our laboratory a 0-10 scale is established.

Grade	Clinical sign	Comment
0	No clinical signs	Normal gait, tail moves and can be raised, tail wraps around a round object if mouse is held at the base of the tail
1	Partially limp tail	Normal gait, tip of the tail droops
2	Paralyzed tail	Normal gait, tail droops
3	Hind limb paresis, uncoordinated movement	Uncoordinated gait, tail limps, hind limbs respond to pinching
4	One hind limb paralyzed	Uncoordinated gait with one hind limb dragging, tail limps, one hind limb does not respond to pinch
5	Both hind limbs paralyzed	Uncoordinated gait with both hind limbs dragging, tail limps, both hind limbs do not respond to pinch
6	Hind limbs paralyzed, weakness in forelimbs	Uncoordinated gait with forelimbs struggle to pull body, forelimbs reflex after pinching, tail limps
7	Hind limbs paralyzed, one forelimb paralyzed	Mouse cannot move, one forelimb responds to toe pinch, tail limps
8	Hind limbs paralyzed, both forelimbs paralyzed	Mouse cannot move, both forelimbs do not respond to toe pinch, tail limps
9	Moribund	No movement, altered breathing
10	Death	

Table 1. Clinical scoring system.

Representative Results

After immunization, mice have to be evaluated daily for changes in weight and clinical symptoms. Disease onset is typically correlated with a reduction of weight which might begin 1-2 days before EAE symptoms are visible. Clinical signs of EAE usually begin between day 9 and 14 post-immunization. As lesions are predominantly localized to the spinal cord in MOG-EAE in C57BL/6 mice, they typically develop predominantly motoric symptoms in a caudal to rostral pattern. An exemplary picture of an immunized mouse with EAE symptoms (score 6) is depicted in **Figure 1A**. Some atypical EAE symptoms might also occur such as rolling in axial-rotary manner, hunched appearance or hypersensitivity which are not reflected within the classical EAE score depicted in **Table 1.** As motor symptoms are the main characteristic in this model, this score does give us a good indication of disease severity. Different monitoring systems (*e.g.* score 0-5 or 0-6) and more complex composite scores assessing different deficits have also been published. Please note that mice with severe symptoms have to be taken out of the experiment

according to the respective institutional animal care and use committee. Mice show generally partial recovery of symptoms over the next 10-20 days. A representative disease course is depicted in **Figure 1B**. There are different time points during EAE which are of interest for assessing outcome parameters. Some typical assays which can often be found are described very briefly. At disease maximum, mice can be evaluated for cytokine production or proliferation after restimulation of isolated immune cells with MOG_{35-55} peptide *in vitro* (MOG recall assay). Immune cells can also be isolated from the brain and spinal cords from mice with EAE symptoms and further processed, *e.g.* by flow cytometry. Histological analysis of spinal cord sections can be performed at disease maximum for lesion load and demyelination, while at later time points markers for neurodegeneration and neuronal cell death might be of interest.

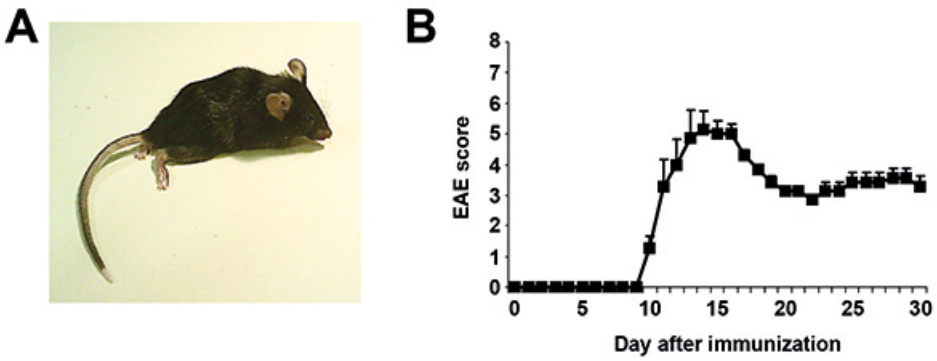

Figure 1. Representative EAE results. A. Representative picture of an immunized C57BL/6 mouse with EAE symptoms (EAE score 6). **B.** Representative disease course after immunization over 30 days. Data are shown as mean and standard error of the mean (n = 5). Click here to view larger image.

Discussion

A multitude of different EAE models with active immunization protocols has been described over the last decades. While rat models have been widely used until recently, mice are now the most popular model organism for EAE research. This development is among others due to the broad and ever increasing repertoire of available transgenic mice. Immunization of C57BL/6 mice with MOG_{35-55} peptide is one of the most widely distributed EAE models and can be considered as a reliable, replicable and well-to-use animal model. In many neuroimmunological laboratories, MOG_{35-55} induced EAE is established as the model of choice while other EAE models are used for more specific experimental questions.

A critical point to consider is the planning of the experimental settings to ensure that EAE experiments are performed methodologically correct. For internal validity, blinded scoring of disease symptoms is highly recommended. Experimental groups should be age-, weight-, and sex-matched and mice should be randomly allocated to treatment groups. Experiments should always be performed in compliance with animal welfare regulations. EAE studies are often underpowered and do not take into account statistical type II errors. Therefore, prior to experiments, sample size calculations should be performed. Necessary group sizes depend on the expected effect size. Consultation of an expert for statistical analysis might be considered before starting EAE experiments.

Some limitations of the protocol need to be kept in mind. Most importantly, interpretation of aEAE data is compromised by the mode of immunization with the use of adjuvant and pertussis toxin which have both additional influence on the immunological reaction. It should also be considered that the MOG_{35-55} EAE model shows mainly a $CD4^+$ T cell driven immunological response. $CD8^+$ T cells and B cells play a less prominent role and alternative protocols should be considered when addressing these cell types. The expected disease course is acute, monophasic and self-limited. Alternatively, a relapsing-remitting disease course can also be achieved in alternative EAE models. An additional important limitation of the protocol is a certain bias towards the immunological component of the MS pathophysiology. During the last years, it has become increasingly clear that MS has a strong neurodegenerative component. The death of oligodendrocytes and neurons results in a progressive accumulation of neurological deficits. It must be taken into account that the EAE model may not be fully suited to address experiment questions concerning neurodegenerative mechanisms of autoimmune inflammation. Alternative animal models with a focus on CNS pathology might be considered – *e.g.* the cuprizone model which compromises toxic demyelination without involvement of the peripheral immune system.

The described protocol be considered as a basic neuroimmunological experimental model and may be modified for other applications. The experimental procedure described above can be easily applied to other EAE protocols by varying mice strains or the type and amount of protein (*e.g.* use $PLP_{139-151}$ peptide and SJL mice for a relapsing-remitting EAE disease course which is especially suited for assessing therapeutic effects on relapses). The described protocol can also be used for adoptive-transfer experiments (passive EAE). In this model, C57BL/6 mice are immunized with MOG_{35-55} peptide and CFA as described above. In contrast, pertussis toxin is not required. After 7-15 days, spleens or lymph nodes are isolated and immune cells are restimulated *in vitro* with MOG_{35-55} peptide and various cytokines prior to transfer into a new group of C57BL/6se mice. These recipient mice develop EAE a few days earlier than upon classical immunization. *In vitro* conditions can be varied for specific immunological questions (*e.g.* polarization into T_H1 or T_H17 cells).

Sometimes, low disease incidence or weak symptoms might be an experimental challenge. Some recommendations for troubleshooting are:

- Disease severity can be varied with different amounts of peptide / mouse.

- Optimal CFA concentration may vary from 1-5 mg/ml. Consider a titration of CFA when establishing the experiments. Please refer to the respective guidelines of the respective institutional animal care and use committee for allowed CFA concentrations as many regulations forbid CFA concentrations exceeding 2 mg/ml.

- Different methods are described for preparing the emulsion. Alternative methods such as vortexing for 1 hr or sonication might be considered if poor emulsification is considered as possible error source.

- Age, gender, season of the year and environmental conditions within the animal facility are important factors that influence EAE susceptibility. It should be ensured that conditions are comparable between independent experiments.

As described above, the mentioned protocol can be used as starting point for adoptive EAE experiments. This model is especially suited for separating peripheral and CNS effects of a genetic phenotype (*e.g.* by transferring encephalitogenic knockout cells into wildtype recipient mice) and for specific immunological questions as the phenotype of the transferred cells can be characterized thoroughly. The latest development in EAE research during the last years are T cell receptor transgenic mice. These mice develop EAE symptoms spontaneously without external influence circumventing the problem of adjuvant inoculation. However, this model requires large amounts of animals for breeding to ensure sufficient group sizes. Evaluation of knockout mice requires crossbreeding prior to EAE experiments in contrast to aEAE. As each mouse develops disease symptoms on a different day, evaluation of novel substances can be rather complicated. Therefore, the value of classical aEAE for neuroimmunological remains unchallenged.

Disclosures

The authors declare no competing financial interests.

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