EXPERIMENTAL MODELS OF MULTIPLE SCLEROSIS
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Edited by

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Dedication

The book is dedicated with love to our families: Dara, Judy, Karen, Irene, Simona and Phillip.
Preface

Experimental Models of Multiple Sclerosis

Multiple Sclerosis (MS) is an enigmatic immune-mediated disease of the central nervous system that affects around 350,000 individuals in the United States. The pathologic hallmarks of this disease are inflammation and demyelination (the destruction of the myelin layer that insulates nerve cell processes, impeding the conduction of neuronal impulses within the brain and spinal cord). The mechanism of demyelination in MS is not completely understood. Immune modulating therapy can delay the progression of MS but there is still no cure for the disease. Thus the development of experimental animal models for MS is among the most powerful tools for studying its pathogenesis and for testing the safety and efficacy of experimental therapies before using these drugs in clinical trials on human patients.

The main experimental models for MS are divided into immune-mediated models or virus-induced models. In immune-mediated models an autoimmune reaction against myelin is induced by the injection of myelin molecules (or portions of it), in combination with immune boosters (Freund’s adjuvant). The prototype of this category is EAE, or experimental allergic (or autoimmune) encephalomyelitis. This model uses a variety of experimental animals including mice, rats, rabbits and even primates. The viral models include several ubiquitous animal viruses such as coronaviruses (mouse hepatitis virus) and enteroviruses (Theiler’s virus) in mice, visna virus in sheep, and distemper virus in dogs. The virus-induced model systems provide both a substrate for studying the pathogenesis of the disease and a hypothetical mechanism for virus-induced autoimmunity in humans.

In this book we have assembled the different experimental models for MS (both immune-mediated and viral). We have asked MS experts to examine aspects of these experimental models highlighting differences and similarities. Each chapter in this book deals with an element of an MS
model: from individual cellular and molecular CNS factors, to cellular and molecular elements of the immune system. Thus the reader is able to read parallel chapters that deal with the same factor in different model systems. The reader of the book will be able to summarize and integrate the different disease components and highlight the common features and the differences that exist in the different model systems. We realize that a book of such magnitude cannot keep pace with all the new knowledge of immunology (in particular new cytokines being discovered), but it offers a conceptual framework. We hope that this book will improve our understanding of the mechanism of demyelination and the pathogenesis of MS. The analysis of common features in the various experimental models of demyelination may also stimulate the development of new ideas about prevention of demyelination and ultimately the treatment of MS.

Ehud Lavi and Cris S. Constantinescu
Editors
Part A

EXPERIMENTAL ALLERGIC (AUTOIMMUNE) ENCEPHALOMYELITIS (EAE)
Chapter A1

EAE: HISTORY, CLINICAL SIGNS, AND DISEASE COURSE

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Abstract: EAE is a useful animal model of autoimmune disease of the central nervous system. In this chapter we discuss the history of EAE, including the original description of the model and the subsequent major developments, and then describe the usual clinical signs and disease course.

Key words: experimental autoimmune encephalomyelitis, encephalitogen, adoptive transfer, active EAE, history, relapses, transgenic models

1. INTRODUCTION

Experimental allergic encephalomyelitis (EAE) is an animal model of autoimmune disease of the central nervous system (CNS). It resembles multiple sclerosis (MS) in many respects, and is used in the laboratory investigation of MS. EAE also serves as a model for the study of organ specific autoimmune diseases in general. EAE was first described over 50 years ago, and it has been a popular and frequently used model. A Medline search of the term identifies more than 5000 citations, with more than 2500 references since 1990. In this chapter, we will review the history of EAE and describe the general features of the model, including the usual clinical signs and disease course.
2. HISTORY

As mentioned in the introduction, there has been an enormous amount of work done in EAE over the last 50 years. In this section, we will briefly summarize the major developments in the field. These include the early studies that led to the establishment of the model, the extensive work done on various antigens, the development of adoptive transfer disease, the development of relapsing EAE, and recent work with EAE in transgenic mice.

2.1 Early Studies

The earliest attempts at producing EAE were efforts to understand the pathogenesis of post rabies vaccination encephalomyelitis. Pasteur's rabies vaccine consisted of a suspension of dessicated spinal cords from rabbits infected with rabies. After a series of injections, occasional patients developed an encephalomyelitis which was distinct from rabies. Early investigators were able to induce a similar encephalomyelitis in rabbits or monkeys by administering repeated injections of neural tissue, thus demonstrating that post rabies vaccine encephalomyelitis probably resulted from the unintentional induction of an autoimmune response against neural antigens [1-3].

In these early experiments the incidence of disease was low, and induction of disease required multiple injections over a period of many weeks. The introduction of complete Freund's adjuvant (CFA) made induction of EAE much simpler and more reliable. CFA consists of a mixture of mineral oil and Mycobacterium made into an emulsion with the antigen, and injecting antigen in CFA usually induces a vigorous and prolonged immune response against the antigen. Several investigators were quick to appreciate the usefulness of this technique. In 1946, Kabat et al published a preliminary report of the induction of EAE in 3 of 4 monkeys using three weekly injections of rabbit brain emulsion in Freund's adjuvant [4]. This was followed rapidly by more definitive reports of similar results in monkeys, rabbits, and guinea pigs [5-8]. In each case the majority of animals developed neurologic deficits after one or a few injections of neural tissue in Freund's adjuvant, and the onset of disease occurred three to four weeks after the first injection. EAE was subsequently induced in many other species, including dogs, cats, rats, mice, sheep, goats, pigs, chickens, and pigeons.

Attempts to induce EAE in mice with antigen in CFA were initially unsuccessful. Olitsky and Yager reported on EAE in mice two years later
The majority of mice developed neurologic symptoms after three to five injections of brain tissue in CFA. The symptoms began about three weeks after the first injection. They noted that their two strains of mice differed substantially in susceptibility to EAE, and future studies verified that susceptibility to EAE varies widely between strains. Subsequently, Lee and Olitsky demonstrated that pertussis vaccine given as a separate injection in addition to antigen in CFA increased the incidence of disease [10]. Munoz and coworkers found that the active ingredient in pertussis vaccine was the toxin, and purified pertussis toxin is now routinely used as an additional adjuvant to induce EAE in mice [11].

2.2 Identification of encephalitogens

The experiments described above were done with homogenate or extract of CNS tissue as the antigen. An early focus of EAE research was identifying the encephalitogenic component of the homogenate. The encephalitogen was present in white matter or myelin rather than gray matter, and appeared to be protein rather than lipid. At the time, there was much controversy over whether brain homogenate contained one or multiple encephalitogens, and over which of the partially purified mixtures prepared with the limited methods available was the relevant encephalitogen. In retrospect, it appears that both of major structural proteins of myelin, myelin basic protein (MBP) and proteolipid protein (PLP), were demonstrated to be encephalitogenic at an early date [12-14].

Subsequent work with MBP demonstrated that the intact protein was not required to induce EAE. Specific fragments of MBP generated by pepsin or trypsin digestion of the whole molecule were encephalitogenic. And in 1970 Eylar et al. induced EAE with a 9 amino acid synthetic peptide corresponding to part of the sequence of MBP [15]. This peptide was as active on a molar basis as the intact molecule. Since then, multiple T cell epitopes for MBP, PLP, and other encephalitogenic proteins have been reported, and synthetic peptides comprising encephalitogenic T cell epitopes are now a standard reagent for inducing EAE.

Recently many other myelin proteins or peptides based on myelin proteins have been demonstrated to be encephalitogenic. Other encephalitogenic myelin proteins include myelin oligodendrocyte glycoprotein (MOG), myelin-associated oligodendrocytic basic protein, and oligodendrocyte specific protein [16-20]. MOG is particularly interesting since it induces relapsing EAE with extensive demyelination. Non-myelin proteins, such as S100β and glial fibrillary acidic protein (GFAP) also cause EAE, but the distribution of the lesions is different than that seen with myelin proteins [21, 22]. Not all CNS proteins will induce EAE. The
myelin protein 2',3'-cyclic nucleotide 3'-phosphodiesterase and the stress protein alphaB crystallin did not induce EAE [19, 23].

2.3 Adoptive or passive EAE

The results discussed above have all been in active EAE, where the disease is induced by injection of CNS antigen in adjuvant. In this model, the induction phase and the effector phase of EAE occur in the same animal. In the induction phase, autoreactive cells are activated and multiply in peripheral lymph nodes in response to the injection of antigen and adjuvant. In the effector phase, the activated autoreactive cells migrate to the CNS and cause autoimmune damage and clinical signs. Another model of EAE is passive or transferred or adoptive disease. In passive EAE, the autoreactive cells specific for CNS antigens are generated in one group of animals, and disease is induced in a second group of animals by transfer of these autoreactive cells. In this way, one can study the effector phase in isolation from the induction phase.

Transfer of disease from EAE induced animals to naïve animals was first achieved by Paterson in 1960 using an elegant experimental design to overcome the difficulty of using outbred experimental animals [24]. Subsequent investigators refined this model by using inbred animals to avoid histocompatibility problems and by stimulating the donor cells in vitro with either mitogen or antigen to increase their encephalitogenic activity [25-27]. CNS antigen-specific T cell lines or clones maintained for prolonged periods in vitro can be used instead of freshly harvested lymph node cells [28]. Passive or transferred EAE is a very reproducible model of EAE since the variability of the induction phase is eliminated.

2.4 Relapsing EAE

Active or passive EAE is usually an acute, monophasic process similar to the human diseases acute disseminated encephalomyelitis or acute transverse myelitis. Investigators interested in multiple sclerosis would prefer a model with relapsing or progressive symptoms to more closely mimic the course of MS, and methods to produce such a model have been described. The induction of relapsing instead of acute EAE depends on the strain of animal, the antigen used, and the induction regimen. A relapsing course has been described in both active and passive EAE, and different regimens have been reported to reliably produce relapsing rather than acute disease [29, 30]. The tendency to relapse varies between strains of mice [31].
2.5 Transgenic models of EAE

The EAE model continues to develop as new methods and techniques become available. One recent development is the use of transgenic mice. Two types of transgenic mice have been constructed for the study of EAE. The first type is T cell receptor (TCR) transgenic mice. These mice express a receptor specific for MBP on all or most of their T lymphocytes [32, 33]. These mice may develop EAE following injection with adjuvant alone, and mice not maintained in sterile pathogen-free environments may develop spontaneous cases of EAE. A second type of transgenic mice was engineered to express a viral protein in oligodendrocytes [34]. Subsequent systemic infection with the corresponding virus resulted in autoimmune CNS damage.

![Figure A1-I. Clinical course of EAE. Representative examples of EAE in SJL mice injected with spinal cord homogenate in CFA and pertussis (see reference 35 for detailed methods). Mice were rated daily for severity of EAE using the scale in Table 1-1. Time in days is on the abscissa, with the day of injection as day 0. Severity of disease is on the ordinate. Mouse A developed monophasic EAE starting on day 12 and resolving by day 23. Mouse B had more severe disease. Mouse C recovered from the initial bout of disease, and then relapsed and had a chronic deficit. Mouse D had severe EAE with rapid onset and died on day 13.](image-url)
3. CLINICAL SIGNS AND DISEASE COURSE

The clinical signs and disease course of EAE vary depending on the strain and species of animal, the inciting neural antigen, the adjuvants, and the timing and dose of antigen and adjuvant. We will describe EAE induced in SJL mice by spinal cord homogenate with mycobacteria and pertussis toxin as adjuvants as a typical example [35]. The first sign is usually ruffled fur and weight loss. One or two days later the animal develops an ascending paralysis. This begins with loss of tail tone and progresses to hind limb weakness and sometimes forelimb weakness. Severely affected mice develop quadriparesis and labored respiration and may die from the disease. The weakness starts about 14 days after injection and worsens over 1 to 4 days. In mice which recover, the symptoms last about 7 days, and relapses after recovery are rare. The clinical course of EAE in representative individual mice is depicted in Figure 1-1.

Severity of disease is rated on an ordinal scale. The scale we use is given in Table 1-1 [31, 35]; other investigators use similar scales with different number of stages or different definitions of stages. We titrate the dose of antigen to minimize the number of mice with stage 5 or 6 disease. Proper veterinary care of severely affected mice is essential.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Clinical deficit</th>
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<tr>
<td>0</td>
<td>no deficits</td>
</tr>
<tr>
<td>1</td>
<td>limp tail or slowed righting response</td>
</tr>
<tr>
<td>2</td>
<td>limp tail and slowed righting response</td>
</tr>
<tr>
<td>3</td>
<td>hind limb weakness with abnormal gait</td>
</tr>
<tr>
<td>4</td>
<td>hind limb paralysis, mobile using forelimbs</td>
</tr>
<tr>
<td>5</td>
<td>hind limb paralysis, forelimb weakness, mobility impaired</td>
</tr>
<tr>
<td>6</td>
<td>moribund</td>
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</table>

The ascending paralysis described above is typical in mice, rats, and guinea pigs. Other species have different clinical courses. Rabbits may have ataxia and gait difficulties, while monkeys may have visual loss, cranial nerve deficits, and ataxia in addition to paralysis.

The acute, monophasic clinical course described above is typical for EAE in rodents. Other disease courses are seen, including a hyperacute form and relapsing or chronic forms of disease. The hyperacute form of disease is characterized by a short latent period between injection and symptom onset and a fulminant course with a high mortality. Chronic or relapsing disease has been described with particular strains of animals and with particular induction regimens.
ACKNOWLEDGEMENTS

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REFERENCES


Chapter A2

INDUCTION OF EAE

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Abstract: Experimental autoimmune encephalomyelitis (EAE) can be induced by two principal approaches. The first is based on direct immunization with autoantigen in combination with different types of adjuvant. The second approach of inducing EAE consists in adoptive transfer of activated T cells that are specific for myelin-associated autoantigens of the central nervous system (CNS). This chapter presents an overview of some useful strategies and protocols by which EAE can be induced, and discusses the pros and cons of various approaches.

Key words: Experimental autoimmune encephalomyelitis, active immunization, adoptive transfer, adjuvant, myelin antigens

1. INTRODUCTION

Methods of inducing experimental autoimmune encephalomyelitis (EAE) have come a long way since initial observations that postvaccinal encephalomyelitis is caused by spinal cord contaminants of rabies vaccine preparations rather than by the inactivated virus itself (1, 2). There are many species susceptible to EAE induction, including mice (3), rats (4), guinea pigs (5), pigs (6) and non-human primates (7). In addition, a human version of accidental EAE, ‘human EAE’, has been described (8, 9).

In 1949, Olitsky and Yager described the induction of experimental disseminated encephalomyelitis in white mice, thus establishing mouse EAE as a model for autoimmune inflammatory diseases of the central nervous system (CNS) (3). EAE in the mouse shares some clinical features with the human disease multiple sclerosis (MS), most prominently paresis/paralysis and ataxia. Depending on the model chosen, EAE can take an acute, a chronic, and/or relapsing-remitting course. Inadvertently induced ‘human
EAE' took a course with striking resemblance to a rare form of MS, acute MS (8, 9). Sensitization with myelin antigens often results in perivenous encephalomyelitis and in some cases, depending on the animal model, in histopathologic changes similar to those seen in MS (9).

There is ample evidence that susceptibility of different animal strains to EAE is linked to MHC class II determinants (10, 11), although non-MHC genes also influence disease expression. Thus, murine EAE can be considered a useful model for some aspects of MS (12).

There are several strategies to induce EAE in animals. EAE can be induced by immunization of animals with mouse spinal cord homogenate (MSCH) or isolated myelin proteins and peptides. Alternatively, EAE is generated by adoptive transfer of lymphocytes that have been activated for myelin antigens in vivo and/or in vitro (11). Both immunized animals as well as mice expressing a transgenic T cell receptor specific for myelin basic protein (MBP) or other myelin antigens can function as a source of T cells suitable for adoptive transfer (13, 14, 15).

Several immunodominant epitopes of myelin components have been characterized, and peptides representing epitopes of MBP, proteolipid protein (PLP), or myelin oligodendrocyte glycoprotein (MOG) are widely used for the induction of EAE. (10, 16-20). As mentioned, the encephalitogenicity of myelin-derived peptides is dependent on the expression of particular MHC class II antigens in the appropriate mouse strain. Thus, induction of disease using PLP peptide PLP_{139-151}, MBP peptide MBP_{87-99} or MOG peptide MOG_{92-106} is suitable in SJL (I-A^*^) mice. The N-terminal peptide MBP_{A31-11} or MBP_{87-99} are suitable in (PL X SJL)F_{1} mice. MOG_{55-55} is routinely used to induce EAE in C57BL/6 mice.

Of note, the human MOG-derived peptide MOG_{97-108}, which has a high binding affinity for the (human) HLA-DR4 MHC class II allele, induces severe EAE in HLA-DR4 transgenic mice (21).

In a recent study, inoculation of myelin-associated oligodendrocytic basic protein and one of its derived peptides, MOBP_{37-60}, were reported to produce severe clinical and histopathologic signs of EAE in SJL mice (22). Moreover, the encephalitogenic potential of synthetic peptides of myelin-associated glycoprotein (peptide MAG_{97-112}) and oligodendrocyte-specific glycoprotein (peptide OSP_{57-72}) has recently been demonstrated in ABH (H-2A^{87}) and SJL mice, respectively, (23).

EAE in the rat is typically an acute paralytic disease from which most animals spontaneously recover. However, also chronic models have recently been described (24). Dominant MBP epitopes in Lewis rats are MBP_{68-86} (25, 26), MBP_{73-86} and MBP_{87-99} derived from guinea pig myelin as well as rat MBP_{73-86} (27). MAG has also been used in the induction of EAE in Lewis
A2. Induction of EAE

rats. In DA rats, MBP_{63-81}, MBP_{79-99}, MBP_{101-120} or MBP_{142-167} can be used for induction of EAE (28). DA rats are also susceptible to PLP-induced EAE.

Recently, EAE was induced in the swine by minipig spinal cord homogenate inoculation. The minipigs presented a monophasic course of disease with spontaneous improvement (6).

Weir et al. demonstrated induction of EAE by transferring bone-marrow-derived dendritic cells presenting MOG_{35-55} into naive C57BL/6 mice (29).

Finally, rabies vaccination in humans has been reported to result in sensitization against neural antigens and lead to acute perivenous encephalomyelitis clinically and histopathologically indistinguishable from some types of EAE in animals or even MS-like lesions (8, 9). Such complications, however, have been very rare.

2. EAE INDUCED BY IMMUNIZATION (ACTIVE EAE) OR BY ADOPTIVE T CELL TRANSFER (PASSIVE EAE, AT-EAE)

2.1 Overview

Active EAE enables the study of both immunization and effector phases in experimental demyelinating disease. Typical immunization protocols require the emulsification of encephalitogens in complete Freund's adjuvant (CFA) containing inactivated mycobacteria such as *Mycobacterium tuberculosis* strain H37RA. Both reagents are obtainable from suppliers such as BD-DIFCO. Antigen concentrations and protocols used for immunizations vary widely between groups and should be adjusted to each laboratory's own conditions.

Direct immunization may complicate the interpretation of experimental data on EAE induction and expression. Due to the severe long-term inflammation caused by the use of CFA, some of the observed effects could be attributed to the mode of immunization rather than to the pathogenic process of inflammation and demyelination in the CNS. The induction of active EAE in some rat and mouse models is significantly enhanced by the administration of heat-killed whole organisms of *Bordetella pertussis* (30, 31) or their derived toxin, pertussis toxin (PTX) (32).

EAE can be induced by transferring sensitized lymph node cells (LNCs) from mice or rats that have been immunized with MSCH, MBP, PLP or other CNS antigens (33-36). Induction of EAE by cell transfer allows the separate study of the effector phase of the disease without the influence of
immunization and inflammation outside the CNS. More importantly, the prime mediators of this type of disease, myelin antigen-specific T cells, can be studied in detail in vitro by standard immunological techniques such as proliferation assays for the determination of antigen specificity, surface marker analysis by flow cytometry, cyto- and chemokine measurements, and T cell receptor sequencing. In vitro analysis of enriched or cloned antigen-specific autoreactive T cells is essential for our understanding of cellular and molecular properties associated with demyelinating disease. In transfer protocols, cells can either be directly transferred after dissection of draining lymph nodes of immunized donor animals or be cultured for various time periods with the appropriate antigen to enrich autoreactive cells.

We describe methods that are routinely used in the laboratory to induce a chronic, often relapsing and remitting paralysis by immunization of mice with PLP$_{139-151}$ or MBP or by adoptive transfer of lymphocytes sensitized to PLP$_{139-151}$ or whole MBP (19, 36, 37).

The method for establishing long-term antigen-specific T cell lines and clones was developed by Kimoto and Fathman in 1980 (38). This technique formed the basis for the induction of autoimmune disease by transferring enriched autoreactive T cell populations. Utilizing this technique, it is possible to establish MBP-specific T cell lines and clones transferring EAE in rats (39) and mice (40). T cell lines specific for MBP or MBP peptides can be established as described (39). The following method for the generation of T cell lines and clones specific for immunodominant and encephalitogenic MBP peptides can be applied to a variety of antigens and in vivo systems (41). Our protocol describes T cell cloning for the N-terminal peptide MBP$_{AcL}$ in PL or (PL x SJL)F1 mice and MBP peptide MBP$_{87-99}$ in (PL x SJL)F1 and SJL mice. In general, this protocol will lead to the isolation of CD4-positive T cell clones with chemotactic activity in vivo (41-43). The optimal concentration for each antigen, e.g. protein or peptide, must be determined both for the immunization of mice and for in vitro primary cultures of LNCs.

### 2.2 Antigenic preparations

As mentioned before, a variety of antigens and antigenic peptides have been described as inducing EAE in the appropriate mouse strains (10, 16-18, 40, 44-47). Most antigens used for the induction of active EAE are suitable for the restimulation of T cells in vitro. Thus, MBP and many other myelin antigens and their derived peptides are capable of inducing T cells that can be cultured in vitro to adoptively transfer EAE.
A2. Induction of EAE

Synthetic peptides are prepared by continuous flow solid-phase synthesis according to the desired sequences. Peptide purity is examined by high-performance liquid chromatography, and peptide identity is confirmed by amino acid composition analysis. Depending on the sequence, the peptide solution may require some pH adjustments before being dissolved in distilled water or phophate-buffered saline (PBS).

After synthesis and before use, peptides should be lyophilized several times to remove volatile organic compounds. Lyophilized peptides should be stored in the cold in a desiccator until use.

MBP for active immunization and for in vitro cell culture can be prepared from guinea pig spinal cords according to the protocol from Deibler et al. (48).

2.3 Active EAE

Mice of the appropriate strains at 6 to 12 weeks of age are immunized by s.c. inoculation with 20 to 400 µg of PLP\textsubscript{139-151}, 400 µg of MBP, or 200 µg of MBP peptide in 0.1 or 0.2 ml of an emulsion of equal volumes of PBS and CFA. To enhance immunization with antigen, killed Mycobacterium tuberculosis strain H37RA is added to the emulsion at a concentration of 0.3 to 4 mg/ml. Injection sites are the regions above the shoulder and the flanks (25 to 50 µl at each injection site). In addition, 200 ng of PTX is injected i.p. or i.v. on the day of immunization and 48 h post immunization. The onset of disease lies usually between 7 and 12 days post immunization. Figure 1 demonstrates a typical EAE course when disease is induced according to the active immunization protocol.

Some older protocols calling for s.c. immunization into the footpads or in the tail base in order to induce active EAE are obsolete due to the fact that the local inflammation can interfere with the gait of animals and hence with EAE scoring.
Figure 1. Active EAE. SJL female mice were immunized with PLP$_{139-151}$ in CFA followed by PTX induction and scored daily for clinical signs of disease.

### 2.4 Generation of PLP- or MBP-specific LNCs

Ten days after immunization, draining lymph nodes are harvested under sterile conditions. The lymph nodes are gently processed and washed through steel mesh until a single-cell suspension is obtained. Cells are then cultured at a concentration of $4 \times 10^6$ cells/ml for 4 days in RPMI 1640 supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 10% fetal calf serum, and 5–15 $10^{-6}$ M PLP$_{139-151}$. Cultures are set in complete medium as described (36, 37) at 2 ml per well of a 24-well tissue culture plate. The generation of MBP-specific LNCs is performed according to the same technique, except that cells are stimulated with 25 µg/ml MBP.

### Isolation and characterization of autoreactive T cell lines and clones

Seven to twelve days after immunization with peptide antigen in an emulsion of PBS and CFA, mice are euthanized, and draining lymph nodes are harvested under sterile conditions. The lymph nodes are gently processed and washed through steel mesh until a single-cell suspension is obtained. Cells ($30 \times 10^6$) are cultured in 5 ml RPMI 1640 supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, $5 \times 10^{-5}$ M 2-mercaptoethanol, 10% fetal calf serum, and antigen (5–15 $10^{-6}$ M). After 4
days of incubation, cells are washed and resuspended in 5 ml of the enriched medium as described above without antigen. Depending on cell growth, the cell culture medium can be supplemented with interleukin-2 (IL-2) or 10% (v/v) concanavalin A (Con A) supernatant as a source of IL-2 (and other growth factors) between stimulations. Con A supernatant is prepared from splenocytes of BALB/c mice by incubating $5 \times 10^6$ cells/ml with 2–5 µg/ml Con A for 24 h. The remaining Con A in the supernatant is removed by stirring it with 2 mg/ml Sephadex G-50 for 1 h. The resulting Con A supernatant is filtered sterile and stored at -70°C until use. T cells are kept in Con A supernatant enriched medium at a concentration of $1 \times 10^6$ cells/ml and restimulated every 14 days using antigen (5–15 $10^6$ M) presented on irradiated (3000 rad) syngeneic spleen cells at a ratio of 1/5 to 1/50 T cells versus antigen-presenting cells (APCs). T cell clones from the MBP-induced T cell lines are derived by the limiting dilution technique. Cells are diluted in medium and distributed in wells of flat-bottom microtiter plates at a minimal concentration of 0.2 cell/200 µl on $5 \times 10^5$ irradiated syngeneic splenocytes as APCs. Resulting clones are maintained by techniques described for the lines. T cell lines and clones generated by this protocol are usually characterized by the expression of CD4, VLA-4, and CD44 cell surface markers (Table 1; 49). Cells proliferate well according to their specific antigen and are often quite heterogeneous with regard to their encephalitogenicity and cytokine production (42, 43).

<table>
<thead>
<tr>
<th>Properties of encephalitogenic T cells</th>
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<tbody>
<tr>
<td>CD4 positive</td>
</tr>
<tr>
<td>Diverse TCR α/β chain gene usage</td>
</tr>
<tr>
<td>High expression of VLA-4</td>
</tr>
<tr>
<td>High expression of LFA-1</td>
</tr>
<tr>
<td>High expression of CD44</td>
</tr>
<tr>
<td>High expression of CD25 (IL-2 receptor α-chain)</td>
</tr>
<tr>
<td>Very low or no expression of L-selectin</td>
</tr>
<tr>
<td>Production of TNF-α/β</td>
</tr>
<tr>
<td>Possible production of IL-4</td>
</tr>
</tbody>
</table>

2.5 Lymphocyte proliferation

Specific recognition of myelin antigens, by sensitized T cells is tested using lymphocyte proliferation assays. Primary proliferative responses of
MBP-specific LNCs are assessed by incubating 2 X 10^5 cells with 100, 50, 25, and 12.5 μg/ml MBP in 200 μl of complete medium per well of a 96-well tissue culture plate. Cultures with no antigen and with 4 μg/ml Con A serve as negative and positive controls, respectively. After 80 h, cultures are pulsed with 1 μCi of [3H]thymidine per well. After 96 h, cells are harvested on glass filters, and radioactivity is determined in a β-counter. All cultures are set up at least in triplicate.

The best time period to examine the proliferation of long-term T cell lines and clones is when the cells are in a resting phase. Thus, 7 to 10 days after antigenic stimulation, cells of T cell lines or clones are tested for their specific proliferative responses to MBP peptides. Ten thousand T cells are cultured in 200 μl in a well of a 96-well tissue culture treated flat-bottom microtiter plate together with 0.5 X 10^6 irradiated (3000 rad) syngeneic spleen cells in the presence of various concentrations of the antigens. A typical concentration range for testing peptide antigens in a proliferation assay includes 0.1, 1, 10, 50, and 100 X 10^-6 M. At the end of a 48-h incubation period, 1 μCi of [3H]thymidine is added. Twelve hours later, cells are harvested and radioactivity is determined in a β-counter. All cultures are set up at least in triplicate.

2.6 EAE induced by adoptive T cell transfer (passive EAE, AT-EAE)

For AT-EAE, 15 X 10^6 to 50 X 10^6 activated autoreactive LNCs are washed in PBS and injected i.v. or i.p. in 200 to 500 μl PBS into naive (PL X SJL)F1 or SJL mice 6 to 12 weeks of age. The severity of EAE is dependent on the number of MBP-specific cells transferred and can be adjusted accordingly, but precise control of clinical EAE scores is very difficult to achieve.

Additional injections of PTX on the day of transfer and 48 h later have been recommended but may precipitate an exacerbative course of disease or even prevent optimal disease induction. In some cases, whole-body irradiation of mice with 350 rad facilitates the transfer of EAE by weakly encephalitogenic T cell clones.

Figure 2 demonstrates EAE induced by adoptive transfer as described above.
A2. Induction of EAE

5-0 4-U 3-W 2-W C2-0,1; 2~ 2; 35 ~; 4~ 4;

Days Post Transfer

Figure 2. AT-EAE. SJL mice were injected i.p. with 36 X 10^6 LNCs/mouse and clinical disease scores were recorded daily.

3. CLINICAL MANIFESTATION AND EVALUATION OF DISEASE

The clinical and histopathologic signs of EAE can be quantified according to various schemes. We evaluate all animals for clinical signs of EAE using a clinical scoring scale ranging from 0 to 5 presented in table 2. Scoring is performed by an examiner blinded to the experimental protocol and by the same observer throughout the experiment. Mean clinical scores at separate days are calculated by adding the scores of individual mice and dividing the sum by the number of mice in each group.

Table 2

<table>
<thead>
<tr>
<th>EAE score</th>
<th>Clinical disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No clinical disease</td>
</tr>
<tr>
<td>1</td>
<td>Tail weakness</td>
</tr>
<tr>
<td>2</td>
<td>Hemi- or paraparesis (incomplete paralysis of one or two hindlimbs)</td>
</tr>
<tr>
<td>3</td>
<td>Hemi- or paraplegia (complete paralysis of one or two hindlimbs)</td>
</tr>
<tr>
<td>4</td>
<td>Paraplegia with forelimb weakness or paralysis</td>
</tr>
<tr>
<td>5</td>
<td>Moribund or dead animals</td>
</tr>
</tbody>
</table>
The onset of actively-induced EAE lies usually between 7 and 12 days post immunization. Maximal clinical severity is often reached between day 9 and day 14, followed by a remitting disease course, although relapses can occur as shown in figure 1. In contrast, transfer of myelin antigen-specific T lymphocytes in the mouse rarely results in a full recovery and tends to leave long-lasting neurological deficits. More severe forms of the disease are associated with an earlier onset, a higher disease incidence, higher disease scores, a slower recovery, and a more synchronized course in the different animals. A significant drop in body weight usually precedes other clinical signs of EAE for about 1 to 2 days. The onset of clinical disease depends on the number and encephalitogenic capacity of the transferred cells.

Disease starts 6 to 12 days after transfer of encephalitogenic cells. Typically, maximal clinical severity is reached between day 8 and day 14 after transfer. Disease course and duration vary according to the animal strain used and the selection cycle of encephalitogenic cells. In primary T cell transfers, a relapsing-remitting disease course over a long time period can be observed, whereas T cell-line- or clone-mediated EAE is characterized by a monophasic disease course with lasting neurological deficits.

3. DISCUSSION

EAE in the mouse is characterized by perivascular inflammatory demyelinating lesions in the CNS and an immunogenetically determined susceptibility. Clinical expression of the disease is obvious by neurological signs and can be easily quantified. The immune system of the mouse is extensively studied, and immune reagents are readily available, making mouse EAE an animal model for the study of some aspects of inflammatory demyelinating disorders of the CNS.

The preparation of T cell lines or clones is obviously much more labor-intensive than mixing myelin antigens in CFA. Why then use passive EAE when active EAE is so much easier? Our laboratories are using routinely passive EAE for several reasons. Passive EAE allows the separate study of immunization and effector events in the pathogenesis of the disease. This procedure avoids local and systemic side effects of adjuvant-based immunizations. Treatment protocols could interfere with the efficacy of autoantigen immunization rather than reflect the effects on the disease process. Finally, cells that mediate EAE in vivo are accessible to characterization and treatment in vitro. The disadvantage of the approach of