

Chapter 11

Animal Models of Multiple Sclerosis

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Summary

Since its first description, experimental autoimmune encephalomyelitis, originally designated experimental allergic encephalitis (EAE), has been proposed as animal model to investigate pathogenetic hypotheses and test new treatments in the field of central nervous system inflammation and demyelination, which has become, in the last 30 years, the most popular animal model of multiple sclerosis (MS). This experimental disease can be obtained in all mammals tested so far, including nonhuman primates, allowing very advanced preclinical studies. Its appropriate use has led to the development of the most recent treatments approved for MS, also demonstrating its predictive value when properly handled. Some of the most exciting experiments validating the use of neural precursor cells (NPCs) as a potential therapeutic option in CNS inflammation have been performed in this model. We review here the most relevant immunological features of EAE in the different animal species and strains, and describe detailed protocols to obtain the three most common clinical courses of EAE in mice, with the hope to provide both cultural and practical basis for the use of this fascinating animal model.

Key words: Experimental autoimmune encephalomyelitis, Multiple sclerosis, Disease induction.

1. Introduction

Experimental autoimmune encephalomyelitis (EAE) is possibly the best animal model to study autoimmune diseases and in particular demyelinating diseases of the central nervous system (CNS) such as multiple sclerosis (MS). Since the first classical studies of Rivers (*1*), in monkeys immunized with CNS homogenate, EAE has been an invaluable tool for dissecting mechanisms of the immune response against self-antigens within the CNS, as well as to test new therapies for the treatment of autoimmune diseases. An autoimmune response leading to EAE in susceptible

species can be obtained by means of active immunization with CNS proteins and by passive transfer of T lymphocytes reacting against myelin antigens to syngeneic recipients. The role of T lymphocytes in EAE was first demonstrated by Paterson who succeeded in transferring disease by means of T cells from immunized animals (2). Since then, many researchers have attempted to characterize the role of T cells in EAE. Over the years, it became clear that activated CD4⁺ T cells mediate EAE upon recognition of the target antigen bound to class II molecules of the major histocompatibility complex (MHC) (3). Encephalitogenic T cells can be retrieved from the blood of immunized as well as naive animals, supporting the concept that auto-aggressive lymphocytes are part of the natural immune repertoire (4, 5).

Several species and strains have been utilized including mice, rats, and guinea pigs. Virtually all mammalian species are susceptible to EAE as long as they are properly immunized. The clinical, pathological, and immunological picture of autoimmune models of demyelination depends upon the mode of sensitization, the nature of the immunogen, and the genetic background of each species and strain.

Modes of sensitization include the route of immunization, primarily subcutaneously, and the use of immunogens emulsified with an equal volume of complete Freund's adjuvant containing *Mycobacterium tuberculosis* to create an antigen depot. Boosts with *Bordetella pertussis* are often used to help in opening the blood-brain barrier (BBB).

Whole myelin homogenate as well as distinct myelin proteins, including myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), and proteolipid protein (PLP), have been used to induce EAE in different species. The role of MBP, an abundant hydrophilic myelin protein, was first characterized by Ben-Nun and colleagues, who successfully induced EAE by transferring MBP-specific T-cell lines to naive Lewis rats (6). Passive transfer studies also elucidated the role of other myelin antigens, including PLP (7) and MOG (8). MOG is a minor glycoprotein exposed on the surface of the myelin sheath and is the target of both humoral and cellular immune responses. The importance of the humoral response to MOG was first demonstrated by Livingston who showed the presence of extensive demyelination following the injection of anti-MOG auto-antibodies into animals with T-cell-mediated EAE (9). Other encephalitogenic proteins include a lipidated form of MBP (10) as well as nonmyelin auto-antigens such as the astrocyte-derived calcium binding protein S100 β (11).

Depending upon the species, the antigen and the mode of sensitization, EAE may have a monophasic acute course (Fig. 1a), as well as a chronic relapsing (Fig. 1b) and even a primary progressive (Fig. 1c) course that mimics human MS. The classical

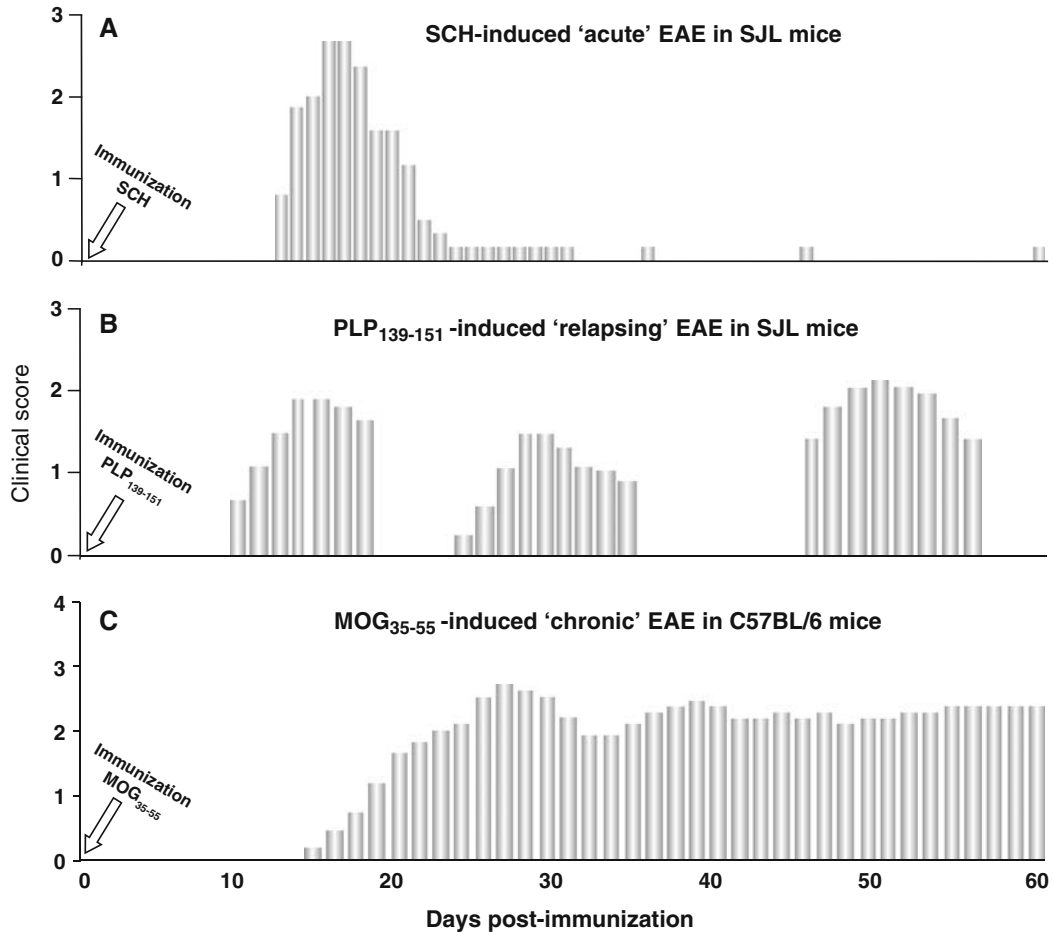


Fig. 1. EAE clinical courses.

picture of acute EAE is characterized by perivascular inflammation mainly represented by CD4⁺ and CD8⁺ T lymphocytes and macrophages within the spinal cord and less consistently in the brain. Nevertheless, the manipulation of the above-mentioned factors can lead to a wide spectrum of neuropathological patterns inclusive of demyelination, remyelination, gliosis, loss of axons, and in certain species also necrosis (12).

1.1. EAE in Inbred Species

Olitsky was the first to establish EAE in mice (13) and since then, thousands of scientists have used inbred rodents as the most suitable species for EAE studies. EAE has been successfully induced in guinea pigs (14) as well as in several strains of rats and mice. Some mice strains such as SJL/J develop a relapsing–remitting disease following active immunization with spinal cord homogenate

(SCH) (15). Pathology shows the presence of mononuclear cell infiltrates within the CNS and demyelination. A similar disease can also be obtained by the adoptive transfer of MBP-specific T lymphocytes (16). In this H-2^s strain, encephalitogenic T cells recognize few epitopes of MBP concentrated within the sequence 89–101, preferentially using the T-cell receptor (TCR) V β 17 segment (17). A more restricted response to encephalitogenic determinants of MBP has been reported for H-2^u mice susceptible to EAE such as PL/J and B.10.PL. Both encephalitogenic and non-encephalitogenic T cells recognize the N-terminal peptide Ac1-9 utilizing the same TCR V β 8.2/V α 2 (or V α 4) gene combination (3). Interestingly, the same TCR gene segments are characteristic of the T-cell response to MBP in Lewis rats (18). In Lewis rats, EAE induced either by active immunization or by passive transfer of T cells is an acute monophasic disease mostly characterized by inflammation (19). In this species, EAE is mediated by CD4⁺ encephalitogenic T lymphocytes specific for the peptide 68–88, in the context of the class II molecule RT1.B¹ (20), which rapidly home and persist at the site of inflammation (21). The dominant use of a restricted TCR repertoire has been successfully exploited in designing immunospecific therapies by means of anti-V β monoclonal antibodies (22) and active vaccination with epitopes of the encephalitogenic TCR molecule (23). Nevertheless, the presence of an epitope dominance, as well as limited TCR usage within the T-cell response to MBP, seems to be confined largely to the early phases of EAE and remains controversial in humans. Later stages of EAE are characterized by a more diverse recognition of previously cryptic determinants inside the MBP molecule (intramolecular spreading) and within other CNS antigens (intermolecular spreading) (24). The appearance of a diverse T-cell repertoire is further confirmed by the presence, in the spinal cord of rats with EAE, of a heterogeneous V β population during the recovery phase of disease (25). This may result from apoptosis of encephalitogenic T-cell clones (26) followed by secondary recruitment of activated cells from the recirculating T-cell pool specific for minor antigens.

Recently, a chronic relapsing form of EAE has been successfully induced in Biozzi (AB/H) mice by immunization with SCH (27) or MOG (28). In this species, pathology shows the full spectrum of lesions seen in MS including sharp demyelination and remyelination. Another useful model was recently described in DA rats which are characterized by a chronic relapsing course, inflammation, and demyelination (29).

In contrast to the rather restricted response to MBP, a diverse recognition of determinants within other myelin antigens, such as PLP (30) and MOG (28), seems to occur in most rodents. Although as a general rule for all inbred species it appears that strains of different haplotype react with different epitopes of myelin antigens.

The presence of encephalitogenic T cells reacting against self-antigens in naive animals is of remarkable conceptual importance and supports the fact that autoaggressive T cells escaping thymic deletion are maintained within the normal circulating T-lymphocyte pool (4, 5). A possible explanation for self-reactive T cells escaping negative selection has been recently clarified in an MBP-/- transgenic model in which endogenous MBP inactivated high avidity clones reacting against the immunodominant epitope making that determinant appear cryptic (31).

Thus, other factors are also necessary to cause an autoimmune disease. The genetic background is a major factor conferring susceptibility to EAE and a number of murine loci have already been identified (32). Both MHC and non-MHC genes have been reported to control the development and severity of EAE (33). The role of environmental factors has been elegantly elucidated by Goverman and colleagues, who created transgenic mice expressing an MBP-specific TCR which spontaneously developed EAE only when challenged with microbial stimuli (34). In a similar model, the complete cohort of anti-MBP TCR transgenic mice, deficient for mature T and B cells, developed spontaneous EAE, suggesting that other cells may have a protective role counteracting with encephalitogenic cells (35). A protective and/or regulatory role has been claimed for almost all cells involved in the immune response including CD4⁺ T cells (36), CD8⁺ (37), CD4⁻ CD8⁻ (38), macrophages (39), B cells (40), $\gamma\delta$ T cells (41), and NK cells (42).

The environment exerts a major effect on EAE, leading to the activation of potentially autoaggressive T cells which consequently home to the brain and induce disease (43). Activation state is the necessary prerequisite for T cells to migrate through the BBB irrespective of their antigen specificity. Activation of myelin-specific T lymphocytes in the peripheral compartment can occur through a mechanism of molecular mimicry (44) and by stimulation with microbial superantigens (45). Other theoretical possibilities such as activation of T cells carrying two sets of receptors, one specific for a foreign protein and another for a self-antigen, has never been demonstrated to play a role in autoimmunity (46). Migration through the BBB requires the involvement of adhesion molecules on both T cells (LFA-1 and VLA-4) and endothelial cells (ICAM-1 and VCAM-1) (47). Following migration through the BBB, neuroantigen-specific CD4⁺ T cells are reactivated in situ by fragments of myelin antigens presented in the framework of MHC class II molecules on the surface of local antigen-presenting cells including macrophages, microglia, and less efficiently also by astrocytes (48). These events are associated with the release of proinflammatory cytokines leading to the up-regulation of MHC molecules on a variety of resident, antigen-presenting cells (49). The kinetics of cytokines along the EAE clinical course suggest the role of

T-helper 1 (Th1) cytokines such as TNF- β , IL-12, TNF- α , and IFN- γ before and at the peak of disease. The recovery phase correlates with Th2 cytokines such as TGF- β , IL-10 and possibly IL-4 (50). The onset of overt inflammation maintains also endothelial activation and leads to a second wave of inflammatory recruitment, including T cells, macrophages that result in tissue damage by means of TNF- α (51), oxygen and nitrogen intermediates, perforin, and complement (52) and demyelinating antibodies (9).

Thus far, no consistent evidence differentiates between activated MBP-specific T lymphocytes with no encephalitogenic capabilities and their pathogenic counterparts, in spite of identical growing and stimulation conditions, sharing of epitope specificity and MHC restriction. Some studies suggest that differences in encephalitogenicity correlate with a predominant Th1 cytokine profile (53), their brain homing capacity (54), and the ability to mediate a delayed type hypersensitivity (DTH) response (55). Moreover, encephalitogenic T cells, despite their CD4⁺ phenotype, have been demonstrated to be cytotoxic for cells, such as astrocytes, presenting myelin antigens in an MHC-restricted manner (56). On the other hand, it has been suggested that nonencephalitogenic T cells can initiate autoimmune regulatory mechanisms through the production of IL-3 (57). It has been postulated that, at least in Lewis rats, encephalitogenicity of MBP-specific T cells correlates with the cytokine profile which depends on the MHC haplotype of the strain (58).

A rather simplistic picture of the EAE cytokine network would suggest that TNF- α , TNF- β , IFN- γ , and IL-12 (proinflammatory cytokines) have a disease-promoting role, while TGF- β , IL-10, and possibly IL-4 (anti-inflammatory cytokines) may protect from disease. Although a detailed analysis of the current literature on this topic is far beyond the scope of this chapter, an enormous amount of data states that the real picture is much more complicated. Several factors influence the cytokine profile of effector and regulatory cells in EAE and, therefore, the final outcome of the immune response within the target organ. These include the age of the animal (59), nature of antigen-presenting cells (60, 61), local cytokine micro-environment (62), selective engagement with co-stimulatory molecules (63), interaction with altered forms of the immunizing antigen (64), and the route of immunization (65).

Based on the hypothesis that Th1 cytokines play a promoting effect on autoimmunity while Th2 cytokines may have a protective role, immune deviation toward a Th2 profile has been exploited for successfully treating EAE by administration of anti-inflammatory cytokines (66), altered peptide ligands (64), monoclonal anti bodies (MAb) affecting B7/CD28 interactions (63), anti-inflammatory cytokines (67, 68), and by induction of oral tolerance (65).

Despite the success of most of the experimental treatments targeting Th1 cytokines, the Th1 vs. Th2 dichotomy underscores the complexity of interactions that lead to reciprocal cross-regulation of Th1/Th2 responses. This has been dramatically elucidated by the severe aggravation of primates EAE due to an enhanced Th2 response occurring after discontinuation of treatment for immune deviation (69). Moreover, conflicting results arise from the utilization of genetically manipulated mice either lacking or over-expressing cytokines, as is the case in a number of TNF- α studies which demonstrated a different clinical phenotype depending on the experimental conditions. For example, over-expression of TNF- α in transgenic mice leads to spontaneous inflammation and demyelination within the CNS (70), while TNF- α -deficient knock-out mice can still develop EAE, thus challenging the role of this cytokine in EAE pathogenesis (71). Surprisingly, in a recent study, MOG-immunized, TNF-deficient mice developed a severe EAE but were remarkably ameliorated by the administration of TNF, also possibly supporting a protective role for this cytokine (72). Targeting cytokine genes has helped to elucidate the role of other cytokines, such as INF- γ (73), IL-4 and IL-10 (74), nitric oxide (75), and also on Fas/Fas-ligand and perforin pathways (76). Nevertheless, it must be kept in mind that results of genetic manipulation result in experimental conditions that only partially represent the in vivo situation and that apparently underscore the redundancy of the cytokine system.

Overall, the deep knowledge of the immunogenetics of inbred species, the possibility of successfully manipulating it, together with their accessibility and costs, still make these species the first choice for studies on autoimmune diseases of the CNS.

1.2. EAE in Outbred Species

EAE in nonhuman primates represented the first experimental model for demyelinating diseases of the CNS (1). The recent advances in housing and handling techniques, knowledge of primate anatomy, immunology, and genetics and the compatibility of most of human reagents and diagnostic techniques have sparked wide interest on EAE in these species. A unique advantage of monkeys arises from their outbred condition that closely resembles the human status. Moreover, the transfer of immunocompetent cells in outbred primates is allowed by the possibility of crossing the trans-species barrier among closely related species (77) and by the natural bone marrow chimerism in some others (5). Therefore, in primates it is possible to elucidate the role of pathogenic cells by means of passive transfer experiments in a polymorphic setting. Recently, EAE has been induced in the common marmoset *Callithrix jacchus*, a unique primate species that develops in utero as genetically distinct twins or triplets sharing bone marrow-derived elements through a common placental circulation (78). It has been recently demonstrated

that *C. jacchus* TCR genes are extensively conserved (79) and that MHC class II region genes, despite a relatively low polymorphism, encode the evolutionary equivalents of the HLA-DR and -DQ molecules (80). A fully demyelinating form of EAE has been induced either by active immunization with whole myelin (78), myelin oligodendrocyte glycoprotein (MOG) or by MBP followed by administration of MOG-specific antibodies (81). Passive transfer experiments have demonstrated that encephalitogenic MBP-specific T cells are part of the normal marmoset repertoire (5). Similarly to humans, MBP-reactive T cells recognize different determinants by means of a diverse TCR repertoire (Antonio Uccelli, personal communication). Pathology of EAE induced with whole myelin or MOG is characterized by perivascular inflammation with conspicuous primary demyelination whose topography correlates with MRI abnormalities (82). On the contrary, immunization with MBP or passive transfer of MBP-reactive T cells leads to mild inflammation and no demyelination (5, Mancardi and Uccelli unpublished results). A complex role of cytokines, possibly released by activated T lymphocytes and macrophages during the immune response, is suggested by the high expression of CD40 and CD40-ligands in marmoset active lesions (83). The role of Th1 cytokines has been demonstrated by the prevention of disease following treatment with the cAMP-specific type IV phosphodiesterase inhibitor Rolipram (84). On the other hand, the ambiguous role of Th2 cytokines was highlighted by the enhancement of EAE occurring after discontinuation of a MOG-based tolerization treatment due to an enhanced proliferative and antibodies response to the antigen. Hence, it is likely that Th2-like T cells play a different role, protecting or favoring autoimmunity, under different conditions (69).

EAE has also been induced in macaques by immunization with myelin or MBP emulsified in complete adjuvant (85). The EAE course is primarily hyperacute or acute, often with a lethal outcome, and is characterized by intense inflammation associated with hemorrhages and necrosis resembling acute disseminated encephalomyelitis (86). The association of EAE susceptibility with an MHC class II allele (87), the presence of myelin-reactive T cells correlating with the course of EAE (86), the beneficial effect of anti-CD4 antibodies on EAE outcome (88) and the possibility of inducing a mild form of EAE by adoptive transfer of MBP-specific T cells from unprimed animals (89) all provide strong evidence that T cells play a central role in this model. At the moment, the major advantage of a nonhuman primate EAE model for human MS resides in the molecular and functional organization of the primate immune system leading to the possibility of evaluating the safety and efficacy of biological molecules as therapy for MS.

1.3. Conclusions

EAE induced in mice and nonhuman primates represents a valuable tool to investigate novel therapeutic approaches in human MS, characterized by neural tissue damage occurring in the context of a very complex immune-mediated inflammatory reaction. In the following sections, we will describe the protocols to obtain (a) acute monophasic EAE (**Fig. 1a**) induced in SJL mice by immunization with SCH; (b) relapsing–remitting EAE (**Fig. 1b**) induced in SJL mice by immunization with PLP139–151; and (c) chronic-progressive EAE (**Fig. 1c**) induced in C57BL/6 mice by immunization with MOG35–55.

2. Materials

2.1. SCH Preparation

1. 10-ml Glass syringes.
2. 21-Gauge needles.
3. Surgical instruments for mouse dissection.
4. 4. 70- μ m Falcon cell strainer.
5. Lyophilizer.

2.2. EAE Induction

1. 10-ml Glass syringes.
2. 30-ml Glass tubes (corex).
3. 21-Gauge needles.
4. Incomplete Freund's Adjuvant (IFA) (Sigma).
5. Heat-inactivated, lyophilized *Mycobacterium tuberculosis* strain H37R (Beckton Dickinson, formerly DIFCO).
6. 1-ml Syringes with removable 25-gauge needle.
7. MOG, residues 35–55 (MEVGWYRSPFSRVVHLYRNGK), or proteolipid protein (PLP) residues 139–151 (HSLGKALGHPDKF) purity over 95% (*see Note 1*), or SCH (*see above*).
8. Pertussis toxin (pancreatic islet activating) from *Bordetella pertussis* (Sigma).
9. Female C57BL/6 or SJL mice (depending on the antigen used), 18–20 g (6–8-weeks old).

3. Methods

3.1. SCH Preparation

1. SCH is prepared according to Levine and Sowinski (90). Using surgical scissors, take out cervical, thoracic, and lumbar column from sacrificed mice.

2. Firmly mount the 21-gauge needle on the 10-ml syringe filled with saline and flush out the spinal cords from the excised vertebral columns.
3. Homogenize the tissue by straining through the 70- μ m Falcon cell strainer using a syringe piston.
4. Weigh the crude SCH and add saline 4:1 (w/v; es. 1,000 mg spinal cords with 250 μ l saline).
5. Lyophilize according to the standard protocol of the available lyophilizer.
6. Store at -20°C .

3.2. EAE Induction

1. *Emulsion preparation.* For the emulsion's final volume, calculate 300 μ l/mouse and at least 2–3 ml excess. In a glass tube, re-suspend the lyophilized antigen (MOG35–55, PLP139–151, or SCH) in 1 $\times$ PBS at concentration indicated in **Table 1** (*see Note 2*).
2. In a second glass tube, prepare a homogeneous suspension of 8 mg/ml *M. tuberculosis* in IFA to obtain CFA. The lyophilized *M. tuberculosis* does not dissolve in IFA. An homogenous suspension can be obtained by careful stirring with a glass syringe, but *M. tuberculosis* will immediately re-precipitate at the bottom of the tube (*see Note 3*).
3. In a third glass tube, mix the antigen, resuspended in PBS, and the CFA in a 1:1 volume ratio. Mix the emulsion by aspirating/expelling with a glass syringe through a 21-gauge needle until it becomes firm, dense, and very creamy (*see Note 4*). This usually takes at least 5–10 min mixing.
4. When the emulsion is ready, fill the 1-ml syringes by aspirating the emulsion through the 21-gauge needle you used to mix it, remove it, place back the original 25-gauge needle (*see Note 5*). Then, swing the syringe in order to have the emulsion towards the piston and push the latter until the dead volume of the syringe is filled. If properly done, this will take you to have 900 μ l emulsion in the syringe, exactly the amount needed for three mice.
5. Inject mice with the emulsion according to **Table 1** (*see Notes 6 and 7*).
6. Inject each mouse intravenously with P.T. re-suspended in 100 μ l of PBS (*see Note 8*), according to the dose and schedule indicated in **Table 1**.

3.3. EAE Clinical Evaluation

1. Clinical score and body weight is recorded daily.
2. Mice will have usually 1–2 g of weight loss in the days preceding clinical onset, which usually occurs, variably, between the 9th and 18th day after immunization.

Table 1
Antigen concentrations and immunization schedules

EAE type	Mouse strain	Antigen used	Antigen concentration	Sites of injection	P.T.	Boost
Acute monophasic	SJL	SCH	27 mg/ml (4 mg/mouse)	The two hind footpads (approx. 50- μ l each) and under the skin of the neck (remain- ing 200 μ l)	400 ng/mouse the day of immuniza- tion and 48 h later	No
Relapsing–remitting	SJL	PLP139–151	2 mg/ml (300 μ g/mouse)	Under the skin of one flank	500 ng/mouse the day of immuniza- tion and 48 h later	After seven days, inject- ing the emulsion on the opposite flank and repeating P.T. the day of the boost and 48 h later
Chronic	C57BL/6	MOG35–55	1.3 mg/ml (200 μ g/mouse)	Under the skin of both flanks and at the tail base, 100 μ l at each site	500 ng/mouse the day of immuniza- tion and 48 h later	No

	0	Healthy mouse
	1	Weak or flaccid tail. (When the mouse, put on a grid like the cage cover, has gait problems = 1.5)
	2	Unsteady gait (ataxia), hind limb paresis, or slow righting reflex
	3	Paraparesis, complete hind limb paralysis
	4	Paraparesis with fore limb involvement
	5	Moribund or death

Fig. 2. EAE clinical evaluation.

3. A nonparametric scale with five steps is used for clinical evaluation (**Fig. 2**). Mice are taken from the cage by the tail and observed from the abdominal side: healthy mice extend their hind limbs very wide when held in this position. An asymmetric position, especially with one of the hind limbs retracted close to the abdomen, may represent an initial sign of disease. A weak tail and an unsteady gait are the signs at onset. To reveal an uncertain gait, it is useful to observe mice walking on a grid, like the cage cover. Healthy mice are quick in movements on a grid almost as on firm ground. Sick mice are unsure, move slowly, and often stumble. In the absence of other signs, we score the uncertain gait on the cage cover as 1.5. Another useful examination is that of the righting reflex. Trying to put healthy mice on its back results in such a quick flipping to the upright position that it is impossible to see the abdomen. A sick mice may have a slow, very slow, to complete impaired righting reflex. In the absence of other signs, we score any grade of impairment of the righting reflex as 2. Paraparesis (score 3) and involvement of fore limbs (score 4) are easily identified (*see Note 9*).

4. Notes

1. Quality of the peptide is the most relevant issue to obtain a good disease. Incomplete incidence or very low severity of EAE are almost invariably related to peptide's quality.
2. In order to avoid waste of material due to weighing procedures, we usually dissolve the lyophilized MOG35–55 or PLP139–151 peptide in 1× PBS at 2 mg/ml, sterilize by filtration on a 0.2- μ m syringe filter (for in vitro immunological assays), and store it at -80°C in 1-ml aliquots for several months, up to 1 year, without loss of encephalitogenicity.
3. To improve the suspension, *M. tuberculosis* can be further triturated in a mortar with a pestel to obtain a more subtle powder, before mixing to IFA.
4. To check the emulsion's quality, let a drop of it fall in water: a good emulsion stays compact without dissolving and without blurred edges.
5. Since the emulsion is very dense, a great pressure has to be applied to push the needed amount out of the syringe. This can lead to the popping off of the needle, and sometimes the emulsion comes out with great pressure and can get in the operator's eyes. Therefore, great care has to be taken to put the 25-gauge needle back to the 1-ml syringe pressing it firmly. Additionally, the connection can be stabilized with a small piece of parafilm.
6. Mice should be housed in an SPF animal facility or, at least, in a clean conventional room since viral, bacterial, and especially parasitic infections can alter the disease course (Roberto Furlan personal observation).
7. Injections under the skin of the flanks is performed at the thoracic level to avoid the possibility to inject the emulsion intraperitoneally: consequent peritoneal inflammation leads to altered gait which is often confused with the clinical manifestation of EAE.
8. Pertussis toxin can be stored at -80°C in aliquots at the final concentration needed, for at least 6 months, with only a minor loss of activity.
9. When we have mice with grade 3 or 4 EAE, we put wet food pellets in the cage to allow paraplegic mice to eat and survive.

References

1. Rivers TM, Sprunt DH, Berry GP (1933) Observations on the attempts to produce acutedissemminated encephalomyelitis in monkeys. *J Exp Med* 58:39–53
2. Paterson PY (1960) Transfer of allergic encephalomyelitis in rats by means of lymph node cells. *J Exp Med* 111:119–135
3. Zamvil SS and Steinman L (1990) The T lymphocyte in experimental allergic encephalomyelitis. *Annu Rev Immunol* 8:579–621
4. Schlusener HJ and Wekerle H (1985) Autoaggressive T-lymphocyte lines recognizing the encephalitogenic portion of myelin basic protein: in vitro selection from unprimed rat T-lymphocyte populations. *J Immunol* 135:3128–3133
5. Genain CP, Lee-Parritz D, Nguyen MH, Massacesi L, Joshi N, Ferrante R, Hoffman K, Moseley M, Letvin NL, Hauser SL (1994) In healthy primate, circulating autoreactive T cells mediate autoimmune disease. *J Clin Invest* 94:1339–1345
6. Ben-Nun A, Wekerle H, Cohen IR (1981) The rapid isolation of clonable antigen specific T lymphocyte lines capable of mediating autoimmune encephalomyelitis. *Eur J Immunol* 11:195–199
7. Yamamura T, Namikawa T, Endoh M, Kunishita T, Tabira T (1986) Passive transfer of experimental allergic encephalomyelitis induced by proteolipid apoprotein. *J Neurol Sci* 76:269–275
8. Linington C, Berger T, Perry L, Weerth S, Hinze-Selch D, Zhang Y, Lu HC, Lassmann H, Wekerle H (1993) T cell specific for the myelin oligodendrocyte glycoprotein (MOG) mediate an unusual autoimmune inflammatory response in the central nervous system. *Eur J Immunol* 23:1364–1372
9. Linington C, Bradl M, Lassmann H, Brunner C, Vass K (1988) Augmentation of demyelination in rat acute allergic encephalomyelitis by circulating mouse monoclonal antibodies directed against a myelin/oligodendrocyte glycoprotein. *Am J Pathol* 130:443–454
10. Massacesi L, Vergelli M, Zehetbauer B, Liuzzi GM, Olivetto J, Ballerini C, Uccelli A, Mancardi GL, Riccio P, Amaducci L (1993) Induction of experimental autoimmune encephalomyelitis in rats and immune response to myelin basic protein in lipid bound form. *J Neurol Sci* 119:91–98
11. Kojima K, Berger T, Lassmann H, Hinze-Selch D, Zhang Y, Gehrmann J, Reske K, Wekerle H, Linington C (1994) Experimental autoimmune panencephalitis and uveoretinitis transferred to the Lewis rat by T lymphocytes specific for the S100 β molecule, a calcium binding protein of astroglia. *J Exp Med* 180:817–829
12. Raine CS (1997) The lesion in multiple sclerosis and chronic relapsing experimental allergic encephalomyelitis: a structural comparison. In Raine CS, McFarland HF, Tourtellotte WW (eds) Multiple sclerosis: clinical and pathogenetic basis. Chapman and Hall, London, pp 242–286
13. Olitsky PK and Yager RH (1949) Experimental disseminated encephalomyelitis in mice. *J Exp Med* 90:213–223
14. Lisak RP, Zweiman B, Kies MW, Driscoll B (1975) Experimental allergic encephalomyelitis in resistant and susceptible guinea pigs: in vivo and in vitro correlates. *J Immunol* 114:546–549
15. Brown AM and McFarlin DE (1981) Relapsing experimental allergic encephalomyelitis in the SJL/J mouse. *Lab Invest* 45:278–284
16. Mokhtarian F, McFarlin DE, Raine CS (1984) Adoptive transfer of myelin basic proteinsensitized T-cells produces chronic relapsing demyelinating disease in mice. *Nature* 309:356–358
17. Sakai K, Sinha AA, Mitchell DJ, Zamvil SS, Rothbard JB, McDevitt HO (1988) Involvement of distinct murine T-cell receptors in the autoimmune encephalitogenic response to nested epitopes of myelin basic protein. *Proc Natl Acad Sci USA* 85:8608–8612
18. Burns FR, Li RX, Shen L, Offner H, Chou K, Vandenbark A, Haber-Katz E (1989) Both rats and mouse T-cell receptors specific for the encephalitogenic determinant of myelin basic protein use similar V α and V β chain genes even though the major histocompatibility complex and encephalitogenic determinants being recognized are different. *J Exp Med* 169:27–39
19. Lassmann H, Brunner C, Bradl M, Linington C (1988) Experimental allergic encephalomyelitis: the balance between encephalitogenic T lymphocytes and demyelinating antibodies determines size and structure of demyelinated lesions. *Acta Neuropathol* 75:566–576
20. Vandenbark AA, Offner H, Reshef T, Fritz RB, Chou C, Cohen IR (1985) Specificity of T lymphocyte lines for peptides of myelin basic protein. *J Immunol* 139:229–233
21. Kim G, Tanuma N, Kojima T, Kohyama K, Suzuki Y, Kawazoe Y, Matsumoto Y (1998) CDR3 size spectratyping and sequencing of spectratype-derived TCR of spinal cord T cells in autoimmune encephalomyelitis. *J Immunol* 160:509–513
22. Zaller DM, Osman G, Kanagawa O, Hood L (1990) Prevention and treatment of murine experimental allergic encephalomyelitis with

- T-cell receptor V β specific antibodies. *J Exp Med* **171**:1943–1955
23. Howell MD, Winters ST, Olee T, Powell HC, Carlo DJ, Brostoff SW (1989) Vaccination against experimental encephalomyelitis with T cell receptor peptides. *Science* **246**:668–670
 24. Lehmann PV, Forsthuber T, Miler A, Sercarz EE (1992) Spreading of T cell autoimmunity to cryptic determinants of an autoantigen. *Nature* **358**:155–157
 25. Offner H, Buenafe AC, Vainiene M, Celnik B, Weinberg AD, Gold DP, Hashim G, Vandenbark AA (1993) Where, when and how to detect biased expression of disease relevant V β genes in rats with experimental autoimmune encephalomyelitis. *J Immunol* **151**:506–517
 26. Bauer J, Wekerle H, Lassmann H (1995) Apoptosis in brain-specific autoimmune disease. *Curr Opin Immunol* **7**:839–843
 27. Baker D, O'Neill JK, Gschmeissner SE, Wilcox CE, Butter C, Turk JL (1990) Induction of chronic relapsing experimental allergic encephalomyelitis in Biozzi mice. *J Neuroimmunol* **28**:261–270
 28. Amor S, Groome N, Linington C, Morris MM, Dornmair K, Gardinier MV, Matthieu JM, Baker D (1994) Identification of epitopes of myelin oligodendrocyte glycoprotein for the induction of experimental allergic encephalomyelitis in SJL and Biozzi AB/H mice. *J Immunol* **153**:4349–4356
 29. Lorentzen JC, Issazadeh S, Storch M, Mustafa MI, Lassman H, Linington C, Klareskog L, Olsson T (1995) Protracted relapsing and demyelinating experimental autoimmune encephalomyelitis in DA rats immunized with syngeneic spinal cord and incomplete Freund's adjuvant. *J Neuroimmunol* **63**:193–205
 30. Greer JM, Sobel RA, Sette A, Southwood S, Lees MB, Kuchroo VK (1996) Immunogenic and encephalitogenic epitope clusters of myelin proteolipid protein. *J Immunol* **156**:371–379
 31. Targoni OS, Lehmann PV (1998) Endogenous myelin basic protein inactivates the high avidity T cell repertoire. *J Exp Med* **187**:2055–2063
 32. Sundvall M, Jirholt J, Yang HT, Jansson L, Engstrom A, Pettersson U, Holmdahl R (1995) Identification of murine loci associated with susceptibility to chronic experimental autoimmune encephalomyelitis. *Nat Genet* **10**:313–317
 33. Weissert R, Wallstrom E, Storch MK, Stefferl A, Lorentzen J, Lassmann H, Linington C, Olsson T (1998) MHC haplotype-dependent regulation of MOG-induced EAE in rats. *J Clin Invest* **102**:1265–1273
 34. Goverman J, Woods A, Larson L, Weiner LP, Hood L, Zaller DM (1993) Transgenic mice that express a myelin basic protein-specific T cell receptor develop spontaneous autoimmunity. *Cell* **72**:551–560
 35. Lafaille J, Nagashima K, Katsuki M, Tonegawa S (1994) High incidence of spontaneous autoimmune encephalomyelitis in immunodeficient anti-myelin basic protein T cell receptor transgenic mice. *Cell* **78**:399–408
 36. Kumar V, Sercarz EE (1993) The involvement of T cell receptor peptide-specific regulatory CD4⁺ T cells in recovery from antigen induced autoimmune disease. *J Exp Med* **178**:909–916
 37. Jiang H, Zhang SI, Pernis B (1992) Role of CD8⁺ T cells in murine experimental allergic encephalomyelitis. *Science* **256**:1213–1215
 38. Kozovska MF, Yamamura T, Tabira T (1996) T-T cell interaction between CD4-CD8- regulatory T-cells and T cell clones presenting TCR peptide. Its implications for TCR vaccination against experimental autoimmune encephalomyelitis. *J Immunol* **157**:1781–1790
 39. Huitinga I, Van Rooijen N, De Groot CJA, Uitdehaag BMJ, Dijkstra CD (1990) Suppression of experimental allergic encephalomyelitis in Lewis rats after elimination of macrophages. *J Exp Med* **172**:1025–1033
 40. Wolf SD, Dittel BN, Hardardottir F, Janaway CA (1996) Experimental autoimmune encephalomyelitis induction in genetically B-cell deficient mice. *J Exp Med* **184**:2271–2278
 41. Kobayashi Y, Kaway K, Ito K, Honda H, Sobue G, Yoshikai Y (1997) Aggravation of murine experimental allergic encephalomyelitis by administration of T-cell receptor $\gamma\delta$ -specific antibodies. *J Neuroimmunol* **73**:169–174
 42. Zhang BN, Yamamura T, Kondo T, Fujiwara M, Tabira T (1997) Regulation of experimental autoimmune encephalomyelitis by natural killer (NK) cells. *J Exp Med* **186**:1677–1687
 43. Naparstek Y, Ben-Nun A, Holoshitz J, Reshef T, Frenkel A, Rosenberg M, Cohen IR (1983) T lymphocyte line producing or vaccinating against autoimmune encephalomyelitis (EAE). Functional activation induces peanut agglutinin receptors and accumulation in the brain and thymus of line cells. *Eur J Immunol* **13**:418–423
 44. Ufret-Vincenty RL, Quigley L, Tresser N, Pak SH, Gado A, Hausmann S, Wucherpfennig KW, Brocke S (1998) In Vivo Survival of Viral Antigen-specific T Cells that Induce Experimental Autoimmune Encephalomyelitis. *J Exp Med* **198**:1725–1738
 45. Brocke S, Gaur A, Piercy C, Gautam A, Gijbels K, Fathman GC, Steinman L (1993) Induction of relapsing paralysis in experimental autoimmune encephalomyelitis by bacterial superantigen. *Nature* **365**:642–644

46. Padovan E, Giachino C, Cella M, Valitutti S, Acuto O, Lanzavecchia A (1995) Normal T lymphocytes can express two different T cell receptor β chains: implications for the mechanism of allelic exclusion. *J Exp Med* **181**:1587–1591
47. Cannella B, Cross AH, Raine CS (1990) Upregulation and coexpression of adhesion molecules correlates with experimental autoimmune demyelination in the central nervous system. *J Exp Med* **172**:1521–1524
48. Myers KJ, Dougherty JP, Ron Y (1993) In vivo antigen presentation by both brain parenchymal cells and hematopoietically derived cells during the induction of experimental autoimmune encephalomyelitis. *J Immunol* **150**:2252–2260
49. Traugott U, McFarlin DE, Raine CS (1986) Immunopathology of the lesion in chronic relapsing experimental autoimmune encephalomyelitis in the mouse. *Cell Immunol* **99**:394–410
50. Olsson T (1994) Role of cytokines in multiple sclerosis and experimental autoimmune encephalomyelitis. *Eur J Neurol* **1**:7–19
51. Selmaj KW, Raine CS (1988) Tumor necrosis factor mediates myelin and oligodendrocyte damage in vitro. *Ann Neurol* **23**:339–46
52. Compston DA, Scolding NJ (1991) Immune-mediated oligodendrocyte injury. *Ann N Y Acad Sci* **633**:196–204
53. Conboy YM, DeKruyff R, Tate KM, Cao ZA, Moore TA, Umetsu DT, Jones PP (1997) Novel genetic regulation of T helper 1 (Th1)/Th2 cytokine production and encephalitogenicity in inbred mouse strains. *J Exp Med* **185**:439–451
54. Baron JL, Madri JA, Ruddle NH, Hashim G, Janaway CA (1993) Surface expression of $\alpha 4$ integrin by CD4 T cells is required for their entry into brain parenchyma. *J Exp Med* **177**:57–68
55. Beraud E, Balzano C, Zamora AJ, Varriale S, Bernard D, Ben-Nun A (1993) Pathogenic and non-pathogenic T lymphocytes specific for the encephalitogenic epitope of myelin basic protein: functional characteristics and vaccination properties. *J Neuroimmunol* **47**:41–54
56. Sun D, Wekerle H (1986) Ia-restricted encephalitogenic T lymphocytes mediating EAE lyse autoantigen-presenting astrocytes. *Nature* **320**:70–72
57. Jeong MC, Itzikson L, Uccelli A, Brocke S, Oksenberg JR (1998) Differential display analysis of murine encephalitogenic mRNA. *Int Immunol* **10**:1819–1823
58. Mustafa M, Vingsbo C, Olsson T, Ljungdhal A, Hojeberg B, Holmdahl R (1993) The major histocompatibility complex influences myelin basic protein 68–86-induced T-cell cytokine profile and experimental autoimmune encephalomyelitis. *Eur J Immunol* **23**:3089–3095
59. Forsthuber T, Yip HC, Lehmann PV (1996) Induction of Th1 and Th2 immunity in neonatal mice. *Science* **271**:1728–1730
60. Krakowski ML, Owens T (1997) The central nervous system environment controls effector CD4⁺ T cell cytokine profile in experimental allergic encephalomyelitis. *Eur J Immunol* **27**:2840–2847
61. Aloisi F, Ria F, Penna G, Adorini L (1998) Microglia are more efficient than astrocytes in antigen processing and in Th1 but not Th2 cell activation. *J Immunol* **160**:4671–4680
62. Falcone M, Bloom BR (1997) A T helper cell 2 (Th2) immune response against non-self antigens modifies the cytokine profile of autoimmune T cells and protects against experimental allergic encephalomyelitis. *J Exp Med* **185**:901–907
63. Kuchroo VK, Das MP, Brown JA, Ranger AM, Zamvil SS, Sobel RA, Weiner HL, Nabavi N, Glimcher LH (1995) B7-1 and B7-2 costimulatory molecules activate differentially the Th1/Th2 developmental pathways: application to autoimmune disease therapy. *Cell* **80**:707–718
64. Nicholson LB, Greer JM, Sobel RA, Lees MB, Kuchroo VK (1995) An altered peptide ligand mediates immune deviation and prevents autoimmune encephalomyelitis. *Immunity* **3**:397–405
65. Weiner HL (1997) Oral tolerance: immune mechanisms and treatment of autoimmune diseases. *Immunol Today* **18**:335–343
66. Racke MK, Bonomo A, Scott DE, Cannella B, Levine A, Raine CS, Shevach EM, Rocken M (1994) Cytokine-induced immune deviation as a therapy for inflammatory autoimmune disease. *J Exp Med* **180**:1961–1966
67. Ruddle NH, Bergman CM, McGrath KM, Lingenheld EG, Grunnet ML, Padula SJ, Clark RB (1990) An antibody to lymphotoxin and tumor necrosis factor prevents transfer of experimental allergic encephalomyelitis. *J Exp Med* **172**:1193–1200
68. Leonard JP, Waldburger KE, Goldman SJ (1995) Prevention of experimental autoimmune encephalomyelitis by antibodies against interleukin 12. *J Exp Med* **181**:381–386
69. Genain CP, Abel K, Belmar N, Villinger F, Rosenberg DP, Linington C, Raine CS, Hauser SL (1996) Late complications of immune deviation therapy in a nonhuman primate. *Science* **274**:2054–2057
70. Taupin V, Renno T, Bourbonniere L, Peterson AC, Rodriguez M, Owens T (1997) Increased severity of experimental autoimmune encephalomyelitis, chronic macrophage/microglial reactivity, and demyelination in transgenic mice producing

- tumor necrosis factor- α in the central nervous system. *Eur J Immunol* **27**:905–913
71. Frei K, Eugster HP, Bopst M, Constantinescu CS, Lavi E, Fontana A (1997) Tumor necrosis factor α and lymphotoxin α are not required for induction of acute experimental autoimmune encephalomyelitis. *J Exp Med* **185**:2177–2182
 72. Liu J, Marino MW, Wong G, Grail D, Dunn A, Bettadapura J, Slavin AJ, Old L, Bernard CC (1998) TNF is a potent anti-inflammatory cytokine in autoimmune-mediated demyelination. *Nat Med* **4**:78–83
 73. Ferber IA, Brocke S, Taylor-Edwards C, Ridgway W, Dinisco C, Steinman L, Dalton D, Fathman CG (1996) Mice with a disrupted IFN- γ gene are susceptible to the induction of experimental autoimmune encephalomyelitis (EAE). *J Immunol* **156**:5–7
 74. Bettelli E, Das MP, Howard ED, Weiner HL, Sobel RA, Kuchroo VK (1998) IL-10 is critical in the regulation of autoimmune encephalomyelitis as demonstrated by studies of IL-10- and IL-4-deficient and transgenic mice. *J Immunol* **161**:3299–3306
 75. Sahrbacher UC, Lechner F, Eugster HP, Frei K, Lassmann H, Fontana A (1998) Mice with an inactivation of the inducible nitric oxide synthase gene are susceptible to experimental autoimmune encephalomyelitis. *Eur J Immunol* **28**:1332–1338
 76. Malipiero U, Frei K, Spanaus KS, Agresti C, Lassmann H, Hahne M, Tschopp J, Eugster HP, Fontana A (1997) Myelin oligodendrocyte glycoprotein-induced autoimmune encephalomyelitis is chronic/relapsing in perforin knockout mice, but monophasic in Fas- and Fas ligand-deficient lpr and gld mice. *Eur J Immunol* **27**:3151–3160
 77. Bontrop RE, Otting N, Slierendregt BL, Lanchbury JS (1995) Evolution of major histocompatibility complex polymorphisms and T-cell receptor diversity in primates. *Immunol Rev* **143**:43–62
 78. Massacesi L, Genain CP, Lee-Parritz D, Letvin NL, Canfield D, Hauser SL (1995) Chronic relapsing experimental autoimmune encephalomyelitis in new world primates. *Ann Neurol* **37**:519–530
 79. Uccelli A, Oksenberg JR, Jeong M, Genain CP, Rombos T, Jaeger E, Lanchbury J, Hauser SL (1997) Characterization of the TCRB chain repertoire in the New World monkey *Callithrix jacchus*. *J Immunol* **158**:1201–1207
 80. Antunes SG, de Groot NG, Brok H, Doxiadis G, Menezes AA, Otting N, Bontrop RE (1998) The Common Marmoset: a new world primate species with limited MHC class II variability. *Proc Natl Acad Sci U S A* **95**:11745–11750
 81. Genain CP, Nguyen MH, Letvin NL, Pearl R, Davis RL, Adelman L, Lees MB, Linington C, Hauser SL (1995) Antibody facilitation of multiple sclerosis-like lesions in a non-human primate. *J Clin Invest* **96**:2966–2974
 82. Hart BA, Bauer J, Muller HJ, Melchers B, Nicolay K, Brok H, Bontrop RE, Lassmann H, Massacesi L (1998) Histopathological characterization of magnetic resonance imaging-detectable brain white matter lesions in a primate model of multiple sclerosis: a correlative study in the experimental autoimmune encephalomyelitis model in common marmosets (*Callithrix jacchus*). *Am J Pathol* **153**:649–663
 83. Laman JD, van Meurs M, Schellekens MM, de Boer M, Melchers B, Massacesi L, Lassmann H, Claassen E, 't Hart BA (1998) Expression of accessory molecules and cytokines in acute EAE in marmoset monkeys (*Callithrix jacchus*). *J Neuroimmunol* **86**:30–45
 84. Genain CP, Roberts T, Davis R, Nguyen M, Uccelli A, Faulds D, Hoffman K, Timmel G, Li Y, Ferrante R, Joshi N, Hedgpeth J, Hauser SL (1995) Prevention of autoimmune demyelination in non-human primates by a cAMP-specific phosphodiesterase inhibitor. *Proc Natl Acad Sci USA* **92**:3601–3605
 85. Shaw CM, Alvord EC, Hruby S (1988) Chronic remitting-relapsing experimental allergic encephalomyelitis induced in monkeys with homologous myelin basic protein. *Ann Neurol* **24**:738–748
 86. Massacesi L, Joshi N, Lee-Parritz D, Rombos A, Letvin NL, Hauser SL (1992) Experimental allergic encephalomyelitis in cynomolgus monkeys. Quantitation of T cell responses in peripheral blood. *J Clin Invest* **90**:399–404
 87. Slierendregt BL, Hall M, 't Hart B, Otting N, Anholts J, Verduin W, Claas F, Jonker M, Lanchbury JS, Bontrop RE (1995) Identification of an Mhc-DPB1 allele involved in susceptibility to experimental autoimmune encephalomyelitis in rhesus macaques. *Int Immunol* **7**:1671–1679
 88. Van Lambalgen R, Jonker M (1987) Experimental allergic encephalomyelitis in the rhesus monkey. Treatment of EAE with anti-T lymphocyte subset monoclonal antibodies. *Clin Exp Immunol* **67**:305–312
 89. Meinel E, Hoch RM, Dornmair K, de Waal Malefyt R, Bontrop RE, Jonker M, Lassmann H, Hohlfeld R, Wekerle H, 't Hart B (1997) Differential encephalitogenic potential of myelin basic protein-specific T cell isolated from normal rhesus macaques. *Am J Pathol* **150**:445–453
 90. Levine S, Sowinski R (1973) Experimental allergic encephalomyelitis in inbred and outbred mice. *J Immunol* **110**:139–43