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Virtues and pitfalls of EAE for the development of therapies for multiple sclerosis

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Experimental autoimmune encephalomyelitis (EAE) is a useful model for aiding the development of new treatments for MS. All therapies approved for MS ameliorate EAE. Two approved medications, glatiramer acetate and Natalizumab, were developed directly from studies in EAE. Several trials are ongoing in MS after success in EAE, including altered peptide ligands of myelin, DNA vaccines and statins. However, EAE has failed to predict the outcome of certain approaches. The reasons underlying such failures are discussed here.

The virtues of EAE for the development of MS new drugs

Although experimental autoimmune encephalomyelitis (EAE) has been spectacularly successful in the development of glatiramer acetate [Copaxone; Teva Pharmaceuticals (www.tevapharm.com)] and Natalizumab [Biogen (www.biogen.com) and Elan (www.elan.com)], tests in EAE predicted poorly the clinical outcomes with tumor necrosis factor (TNF) blockade, for example, and failed to illuminate some inherent toxicities of certain approaches, which were discovered ultimately only in the course of clinical studies. These problems are, in part, due to the lack of diversity in EAE models using inbred strains of mice, various misconceptions about our understanding of the pathophysiology of EAE from knockout mice, the relatively small numbers of animals used in EAE test systems, and the short time interval for testing even 'chronic' therapies in EAE, in which experiments might need to run for years, not weeks or months, to model chronic human therapy. Despite its pitfalls, EAE has been a useful model for predicting success with clinical trials in multiple sclerosis (MS).

Currently, there are several approved therapies for MS, including glatiramer acetate [1–8], mitoxantrone [9–11], various β interferons (IFNs) [12,13] and natalizumab [14–17], which has been withdrawn currently from the market by its manufacturers. Glatiramer acetate is one of the hallmarks of current therapy and was developed based on its ability to modulate EAE [1–8]. The most effective medication to date for the treatment of MS, natalizumab, a monoclonal antibody to α4β1 integrin, was developed based on its ability to block lymphocyte adherence to blood vessels in EAE brains [14–17]. Several treatment strategies in development currently, including statins, which block the activity of the enzyme 3-hydroxy 3-methylglutaryl-coenzyme A (HMG-CoA) reductase [17–21], peroxisome proliferators-activated receptor (PPAR) agonists [22], Laquinimod [Active Biotech Research AB, Sweden (www.activebiotech.com)] [23,24], altered and native myelin peptides [25–31] and antigen-specific DNA vaccination [17,32–35], have proven efficacious when given after the onset of paralysis in more chronic models of EAE (Box 1). These approaches are all in clinical trials in MS at present (Figure 1).

Box 1. Novel therapies for MS developed from their unexpected efficacy in EAE

Certain therapeutic approaches, for example, statins, approved for lowering blood cholesterol, PPAR agonists, approved for the treatment of hypertriglyceridemia, and antihistamines, approved for the treatment of allergy, have shown unanticipated efficacy in treating EAE, and have lead to the discovery of novel mechanisms of action of compounds that have been approved for other indications. The mechanisms of action on EAE of some of these compounds are given here:

Statins
- Reduce expression of inducible MHC II, downregulation of co-stimulation [19]
- Reversal of EAE and clinical paralysis in animal models, induces Th1 to Th2 shift [19]
- Reduction in gadolinium-enhancing lesions in relapsing remitting MS patients in a small clinical trial [21]

PPAR agonists
- Reduce expression of inducible MHC II (PPARγ) [22], down-regulation of co-stimulation [19]
- Reversal of EAE and clinical paralysis in animal models, induces Th1 to Th2 shift (PPARα) [22]

Anti-histamines
- Reversal of EAE and clinical paralysis in animal models, induces Th1 to Th2 shift [19]
- Histamine 1 receptor increased on Th1 T cells reactive to myelin that cause EAE [17,35]

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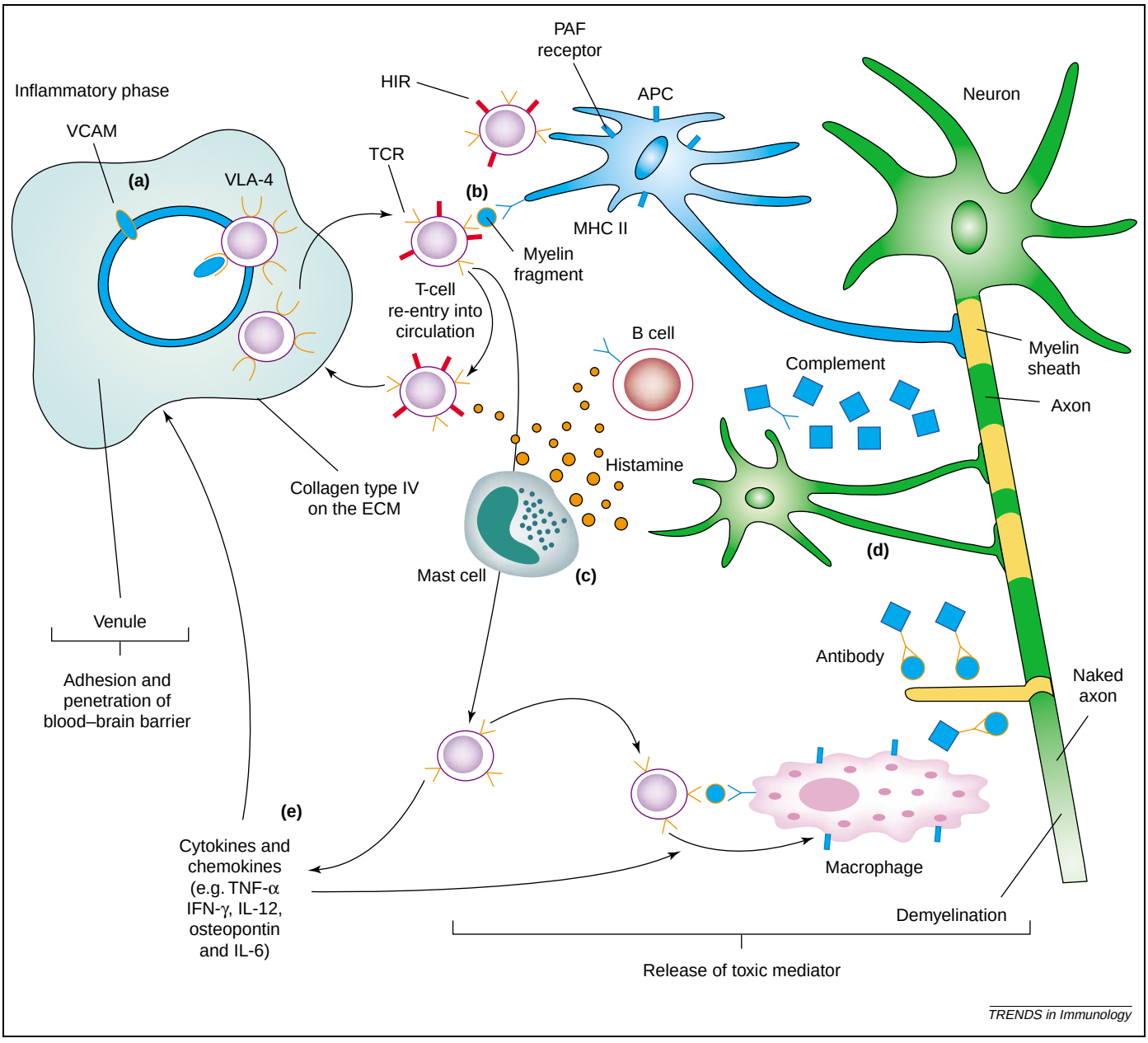

Figure 1. EAE serves as a model for proof of concept of new MS therapies. Interventions at various checkpoints in the pathophysiology of EAE, and presumably MS, are shown. T and B cells penetrate the blood vessel endothelium. **(a)** The key molecule in adhesion is α4β1 integrin on T and B cells. This integrin binds to VCAM and the lymphocytes diapedese, crawling through and penetrating the extracellular matrix. Matrix metalloproteases (MMPs) are crucial for this process. MMPs can be inhibited with IFN-β. Antibodies to α4β1 integrin inhibit the adhesion step. **(b)** Once inside the brain, T cells recognize myelin fragments in association with class II molecules of the MHC. The expression of these molecules, including MHC II and co-stimulatory molecules, such as CD80, CD86 and CD23, can be inhibited by statins and PPAR agonists. APLs and glatiramer can inhibit the interaction of MHC II with T cells. **(c)** Mast cells also have a role in the inflammatory response in MS. In EAE, histamine antagonists and platelet-activating factor (PAF) antagonists can prevent EAE. **(d)** Antibodies to protein and lipid components of the myelin sheath can activate complement, culminating in the production of membrane attack complexes, which damage the oligodendrocyte and lead to the stripping of myelin by activated macrophages. **(e)** Destructive cytokines, such as IL-6, osteopontin, TNF and IFN-γ, amplify the inflammatory response in the brain. Some of these cytokines have Janus-like activities, both inducing pathology but also having key roles in recovery. Modified from Ref. [35].

However, in many notable instances, EAE has not been predictive of clinical toxicities when these approaches have been taken forward into clinical trials. EAE models failed to predict the risk of opportunistic infection, particularly progressive multifocal leukoencephalopathy, seen after chronic blockade of α4β1 integrin [14–17], and also failed to demonstrate the cardiotoxicity seen with Linomide (quinoline 3-carboxamide) [Active Biotech Research AB, Sweden (www.activebiotech.com)] [36,37]. Only after allergic-type hypersensitivity reactions were observed in clinical trials with altered peptide ligands (APLs) was an animal model developed to reveal that such molecules, including APLs and other self-molecules, could cause anaphylaxis [29,38]. EAE is thus particularly valuable for showing *in vivo*, preclinical proof of principle of novel pathways in the pathogenesis of autoimmune disease, however, the model falls short in predicting potential side effects of therapy. One of its major shortcomings is the short-term nature of EAE experiments, lasting several weeks in the acute phase, and only several months in the 'chronic' phase. Current therapy for MS must be considered chronic, extending over years and

decades. The EAE model has failed to detect problems that often arise after months or years of therapy.

The various EAE models and approved drugs for MS

EAE has served as a useful tool for the preclinical testing of new approaches to Th1 autoimmunity. MS is a disease often marked by episodes, with neurological deficits ranging from paralysis to blindness, sensory disturbances and bowel and bladder dysfunction. These episodes last days to weeks and are often followed by periods of remission. Other forms of MS, including primary and secondary progressive MS, do not have these distinctive periods of relapse and remission and the neurological deficits appear and progress. MS often begins in young adulthood and females are affected twice as frequently as males. Thus, MS has several clinical forms, including initial attacks of optic neuritis, episodes of relapsing and remitting paralysis and sensory deficits, and more progressive deterioration without clear relapses or remission. There are EAE models of all of these clinical forms of MS: for example, there is an EAE model for relapsing remitting disease, in which deficits like hind limb paralysis wax and wane after an initial attack [39]. A single T-cell clone reactive to a given region of a myelin protein can induce progressive paralysis leading to death when given in high doses and relapsing and remitting episodes of paralysis when given in smaller doses [40,41]. These T-cell clones are capable of inducing both inflammation in the perivascular white matter and demyelination of the sheath surrounding the neuronal axon [41,42]. Models of EAE reflecting more progressive disease, without clear episodes of relapse and remission, have also been described [43]. A model of pure optic neuritis has been constructed in a transgenic mouse with TCRs recognizing the major encephalitogenic epitope of myelin oligodendroglial glycoprotein (MOG) [44,45]. Models of spontaneous disease in mice made transgenic for crucial human HLA and T-cell (TCR) receptor genes have been described [46]. These various models of EAE, share many features in common with MS (Table 1).

Similarities between EAE and MS are present on many levels [45]. The most important factor in genetic susceptibility in both diseases resides in the MHC. In both diseases, CD4 and CD8 T cells can be found in lesions, including evidence of populations of clonally derived T cells, some reactive to myelin proteins. In most EAE models, CD4 T cells predominate in lesions, whereas in MS lesions both CD4 and CD8 T cells are present. Antibodies and complement are found in lesions in both models. EAE and MS are characterized by damage to the myelin sheath and in both the animal models and in MS there is evidence for axonal degeneration (Table 1).

Those therapies that actually ameliorate or reverse established EAE have shown some promise in the clinic (Table 2). Perhaps most noteworthy has been the development of the random co-polymer of tyrosine, glutamate, alanine and lysine, with the relative concentrations of the amino acids formulated to resemble that of myelin-basic protein (MBP). First invented by Teitelbaum, Arnon and Sela at the Weizmann Institute, the drug was shown to block EAE in a study published in 1971, a full 25 years before the drug was finally approved for treatment of relapsing remitting MS [1,8]. The drug advanced through clinical trials in various models of EAE, including studies in non-human primates, over a period approaching 20 years, and is now an approved drug for multiple sclerosis, based on its capacity to diminish the rate of relapse in MS patients.

Janus-like cytokines and pitfalls of the EAE model

Many scientists interested in developing new therapies for MS criticize the EAE model for its poor record of predicting outcomes in the clinic, especially for those instances when promising therapies indicate that they are beneficial in models of EAE, yet then fail in subsequent clinical trials. They often point to the failure of experiments with EAE to predict how blockade of TNF, with either monoclonal antibodies to TNF or with fusion proteins that block TNF receptor, would work in MS [17,35]. It was hypothesized widely that blockade of TNF would be beneficial in MS, just as it is in rheumatoid arthritis and Crohn's disease. After all, many scientists envision MS, RA and Crohn's autoimmune diseases as mediated by Th1 cytokines, therefore blockade of TNF or its receptor ought to be highly beneficial in MS.

It is important to note that cytokines have multifaceted roles. Thus, TNF is a pathogenic cytokine in rheumatoid

Table 1. Similarities between EAE and MS

Characteristic	EAE	MS
Genetic susceptibility	Strong association with MHC II	Strong association with MHC II
	Females more susceptible in certain strains	Females more susceptible
Environmental triggers	Relapses with earlier infection; superantigens trigger relapses	Association with earlier infection
White matter pathology	Th1 T cells, B cells, CD4 and CD8 T cells, B cells and antibodies to myelin in lesions	Th1 T cells, B cells, CD4 and CD8 T cells, B cells and antibodies to myelin in lesions
	Clonal CD4 and CD8 T cells reactive to myelin components	Clonal CD4 and CD8 T cells reactive to myelin components
	Macrophages	Macrophages
	Microglia	Microglia
	$\alpha 4\beta 1$ integrin	$\alpha 4\beta 1$ integrin
	Complement	Complement
Grey matter pathology	Axonal degeneration	Axonal degeneration
Clinical presentation	Optic neuritis, myelitis, periventricular white matter inflammation	Optic neuritis, myelitis, periventricular white matter inflammation
Clinical forms	Relapsing remitting	Relapsing remitting
	Progressive	Progressive

Table 2. Prevention and reversal of EAE predicts clinical success

Therapeutic	Prevents EAE	Reverses ongoing EAE	Efficacy in MS
Glatiramer acetate	Yes (used with adjuvants)	Not tested, in approval package	Approved for relapsing remitting MS, reduces relapse rate by 30% [6,8]
IFN-β	Yes	Yes	Approved for relapsing remitting MS, reduces relapse rate by 30% [17]
APL to MBP	Yes	Yes	Phase 2b; reduced magnetic resonance activity in Phase 2a at low doses [29]; hypersensitivity after repeated injection at high dose [29]; reports of exacerbation only at high dose [30]
Native peptide for MBP	Yes	Yes	Reduces anti-MBP antibody levels in spinal fluid. Now in Phase 2–3 [17]
Anti-α4β1 integrin, Natalizumab	Yes	Yes	Approved but withdrawn after cases of PML were seen [16]
Statins	Yes	Yes	Early clinical trial with simvastatin showed reduction of activity on magnetic resonance scans [21]
Quinolines	Yes	Yes	Quinoline carboxamide reduced MR activity in Phase 2 but withdrawn due to cardiotoxicity in Phase 3. Later generation versions of these compounds appear not to have this toxicity [37]
Tolerizing DNA vaccine	Yes	Yes	Phase 1–2 trial in progress

arthritis and in Crohn's Disease, whereas it is a growth factor for oligodendroglial cells. Therefore, a cytokine might have both pathogenic and physiologic roles in different contexts. Cytokines have Janus-like properties depending on their context, just as words in language can have opposite meanings depending on their context.

William Safire described Janus-like words that can have opposite meanings in different contexts: 'a term that is its own antonym' [47]. In the following sentence, 'she sanctions your cutting edge work', the word 'sanctions', when used as a verb has positive connotations, implying 'approval' and even 'praise'. In the next sentence, 'she cannot leave her lavish estate because she is under court sanctions', the word 'sanctions' is used as a noun with negative connotations, implying the concept of 'house arrest'. The roles of the cytokines IFN-γ and TNF have demonstrated how predictions made from results in EAE models can go badly awry when taken into the clinic. EAE is ameliorated by IFN-γ, whereas administration of IFN-γ to MS patients worsens disease [17,35,45]. Likewise, the role of TNF in EAE reveals a complicated story, with several papers describing its role as a pathogenic cytokine, and other highly regarded publications pointing to the beneficial role of this cytokine. Clearly, EAE has been a problematic model in this instance. At the time we treat MS, there might often be long established disease, which stands in sharp contrast to the temporal context of EAE, in which disease has been either merely induced or there has been a first attack and then therapy is instituted. Thus, the contexts when drugs are used in EAE might not always reflect the contexts in which they are used in MS.

Conflicting results with TNF in EAE and its failure in MS trials

In the 1990s, several publications showed that blockade of TNF or gene deletion of *TNF* in various EAE models, ameliorated disease. Contradictory publications showed that administration of TNF was actually protective and reversed the exacerbation of EAE seen in TNF knockout mice [48,49]. How did these discordant results arise? The answer lies in three areas: (i) the problem of drug delivery, (ii) the use of knockout models to study autoimmunity and (iii) the reliance on models of EAE that are driven primarily by CD4 T cells.

In EAE, the efficacy of TNF blockers is most pronounced when these fusion proteins that block TNF receptor or the anti-TNF antibodies are injected directly to the brain. This might indicate that TNF blockade would work in MS, if only one could find drugs that would penetrate into the brain itself. Fusion proteins that block the TNF receptor and monoclonal antibodies to TNF are notoriously ineffective at penetrating into the central nervous system (CNS) in patients with MS. TNF blockade by drugs capable of lowering TNF levels within the CNS might still be a viable approach for the treatment of MS, even though monoclonal antibodies and fusion proteins have thus far only exacerbated disease. After all, there is a large body of published literature demonstrating that TNF is a pathogenic cytokine in MS and in EAE.

The evidence is extensive in support of TNF as a pathogenic cytokine in MS: TNF and lymphotoxin (LT) are crucial in the development of EAE and in the human disease, MS [50,51]. Both TNF and LT mRNA and protein are present in the CNS in acute EAE [50]. T-cell clones, reactive to MBP, are more capable of mediating EAE when they produce higher amounts of TNF and LT [52]. Blockade of clinical paralysis in EAE has been successful with anti-TNF antibodies [53,54] or soluble TNF type I receptors [55–57]. Reversal of EAE is seen with altered peptide ligands of MBP that reduce TNF production [25,26]. Reduction of TNF with type I phosphodiesterase inhibitors, such as the antidepressant, Rolipram [Schering AG (www.schering.de)], also leads to the reversal of EAE [58]. TNF is produced in high amounts by glial cells in mouse strains that are susceptible to EAE but not in resistant strains [59]. Demyelination is mediated *in vitro* in oligodendroglial cultures by TNF and LT [60]. Overexpression of TNF in the CNS leads to demyelination [61].

Injection of TNF can trigger EAE relapses [62,63]. All these experiments in EAE reinforce the findings indicating that TNF has a pathogenic role in MS. Finally, TNF and LT are found in demyelinating lesions in the brains of MS patients, and synthesis of TNF in the cerebrospinal fluid in patients with MS has been shown. The level of TNF in cerebrospinal fluid correlates with the severity and progression of the disease [64].

The problem with interpretations of the role of cytokines, such as TNF, from studies of knockout animals has been discussed extensively in an editorial [65]. In many of these knockout animals, lymphoid architecture was distorted or mice were reconstituted with bone marrow after lethal irradiation. These situations are hardly physiological and make interpretations of what might happen in translating the results to humans highly problematic. One of us wrote: 'misconceptions have arisen concerning the interpretation of experiments with these contemporary knockouts. This is especially true when trying to understand the role of critical effector molecules like cytokines, in the development of complex phenotypes, like the paralysis and inflammation seen in EAE. Many of these cytokine molecules have diverse biological activities, and many of the functions of these molecules can be duplicated by other cytokines. Thus, in animals with disrupted or "knocked out" cytokine genes, one may expect many diverse changes in several physiological processes, and one might find that after all is done, that another gene and its product can replace the function of the gene that was disrupted' [65]. 'Often, the genes under study are inactivated during the entire life of the organism, and are inactivated throughout the organism. In these TNF- and LT-deficient mice there is abnormal spleen architecture, blood lymphocytosis, absence of lymph nodes, and functional defects in T cell physiology' [65]. Are these appropriate conditions to study gene function and make conclusions about the role of these genes in autoimmunity?'

A third reason why experiments in EAE fail to predict what would happen with TNF blockade stems from the reliance on certain EAE models. In most EAE models, CD4 T cells have the primary role. EAE models have been devised that are mediated by CD8 T cells [66,67]. In these models anti-IFN-γ reduces disease and TNF blockade has little effect, compared to EAE in models mediated by CD4 T cells, in which anti-IFN-γ worsens disease and TNF blockade produces contradictory results, generally in the direction of improving disease. Thus, certain models of EAE are more predictive than others in demonstrating how administration of a cytokine, such as IFN-γ, or blockade of cytokines, such as TNF, might actually perform in the clinic [66,67]. It is difficult to know *a priori*, which particular model of EAE will be more illuminating, although it is wise to remember that EAE models come in many varieties, and that each model has certain pitfalls.

Once in the clinic, TNF blockade with the monoclonal anti-TNF antibody known as infliximab [Remicade; Johnson and Johnson (www.jnj.com)] or with a TNFRp55–Fc construct, lenercept, which never reached the market, actually increases relapses in MS. The approved anti-TNF drugs for rheumatoid arthritis and Crohn's disease carry warning labels that they could exacerbate MS [17]. Similarly, administration of IFN-γ, which reverses EAE, actually worsens MS [17].

Problems in predicting infectious, metabolic and immunological complications

The story of the rise and fall of natalizumab represents one of the most stunning predicaments in modern medicine. Natalizumab was developed, based on our identification of $\alpha4\beta1$integrin as the crucial molecule involved in the homing of lymphocytes to inflamed brain in EAE. In a collaboration between the Steinman and Yednock laboratories, Stamper Woodruff frozen section assays were used to demonstrate that $\alpha4\beta1$ integrin was the dominant adhesion molecule involved in the homing of lymphocytes to the EAE brain [14,16]. In MS, $\alpha4\beta1$ integrin was demonstrated in lesions along with its receptor vascular cellular adhesion molecule-1 (VCAM-1). At doses of 4 mg kg^{-1}, in rodent studies in EAE induced by pathogenic T-cell clones, monoclonal antibodies to $\alpha4\beta1$ integrin blocks paralysis and brain inflammation in the Lewis rat model of EAE [14,16].

When investigators studied whether antibodies to $\alpha4\beta1$ integrin would block immune surveillance of infections with cytomegalovirus [16] or Borna virus [16], no adverse reactions were seen. In fact, encephalitis caused by Borna virus was ameliorated by an antibody to $\alpha4\beta1$ integrin. The investigators even advocated an antibody to $\alpha4\beta1$ integrin for the treatment of certain forms of encephalitis. Pathology in many forms of encephalitis induced by non-cytopathic viruses is actually due to the inflammation resulting from the immune attack and clearance of the virus, rather than to the virus itself.

Based on these promising preclinical results, a humanized monoclonal antibody, named Natalizumab, to $\alpha4\beta1$ integrin was engineered and taken into the clinic at doses from 3 to 6 mg kg^{-1} monthly, for treatment of relapsing remitting MS. The drug was approved after one year of a Phase 3 clinical trial, which showed a 66% reduction in relapse rate. Two-year results were similar, showing a 66% reduction in relapse rate. However, only three months after the drug received expedited approval by the Food and Drug Administration (FDA), it was withdrawn after three cases of progressive multifocal leukoencephalopathy (PML), two fatal, were seen among the few thousand patients who had taken Natalizumab for two years [16].

How could the preclinical models have predicted this unusual complication? PML is not seen in mice because JC virus (named after a patient with the initials JC), which causes PML, does not infect most species used to test in EAE. Here we have an example of the development of a disease, unique to humans, which could not have been discovered with the EAE model. However, the EAE model was remarkably useful in predicting success for blockade of $\alpha4\beta1$ integrin as a treatment for MS. Even the dose regimen was predicted from the initial landmark clinical study [16].

Metabolic complications of drugs used to treat EAE have rarely been assessed. Linomide, a quinoline derivative, was taken into the clinic after studies in EAE showed striking amelioration of disease and shifts in cytokine profiles from Th1 to Th2. The drug reduced lesion number markedly on magnetic resonance scans when tested in Phase 2 studies. However, a Phase 3 study was terminated abruptly, owing to cardiac toxicity caused by this class of compound. A newer quinoline derivative, Laquinimod, appears to be devoid of this toxicity. Again, detailed toxicology studies are not normally part of studies in EAE, and thus problems, such as cardiotoxicity, are unlikely to be encountered when studying EAE [24,36,37].

Finally, immunological problems have been encountered when translating approaches, such as APLs, from EAE studies to studies in humans. APLs reverse ongoing paralysis effectively in EAE. By interfering with normal signaling through the TCR, APLs reduce TNF and IFN-γ production by myelin-reactive T cells, and induce a Th1 to Th2 shift in cytokines [25,26,28]. *In vivo* administration of anti-interleukin-4 (IL-4) abrogates the effect of APLs [26]. When taken into the clinic, in a placebo-controlled double blind trial, an APL to MBP, given subcutaneously at a dose of 5 mg given weekly for 16 weeks, reduces the number of enhancing lesions on magnetic resonance scans in relapsing remitting patients [29]. There is no increase or decrease in relapse rate. Ten percent of patients had skin site injection reactions, indicative of immediate hypersensitivity reactions. Similar rates of immediate hypersensitivity reactions are seen with glatiramer acetate: no anaphylaxis was seen. A shift to Th2 cytokine production was observed in MBP-reactive T cells [29]. At a dose of 50 mg per week, three of ten patients experienced relapses, with two patients showing an increase in T cells reactive to MBP. The dose response indicated some efficacy at 5 mg and some disease worsening at 50 mg [30].

After immediate hypersensitivity was noted with APL, an animal model of anaphylaxis to myelin proteins was developed. The hypersensitivity reactions are mediated by an IgG1 antibody in mice and can be blocked with antihistamines, such as cyproheptadine, and histamine receptor 1 blockers, such as pyrilamine [38]. Repeated subcutaneous injections of glatiramer also induce anaphylaxis in EAE (L. Steinman, unpublished).

The toxicity of APL actually enables us to reverse engineer a model of anaphylaxis in rodents and to describe, for the first time, anaphylaxis to self [38]. The interplay between animal studies and human studies can thus come full circle, enabling the construction of animal models that might help us to devise new therapies that are devoid of toxicities first encountered in clinical testing in humans.

EAE in balance: a tool for pre-clinical proof of concept, with a record of success

EAE has thus been a versatile model that has lead to the development of highly effective new therapies for MS (Box 1). The model is not without its pitfalls and problems. EAE is certainly bad in predicting toxicities of new drugs because, for the most part, toxicological experiments are not performed. It is also notoriously deficient in detecting potential infectious complications because most experiments are run on insufficient numbers of animals for too short a period of time in animal facilities that are designed to prevent the spread of infectious pathogens. The animals tested are often inbred, so genetic differences in the group tested are negligible and not reflective of what one might see in outbred human populations. Nevertheless, the EAE models are rapid, and can quickly give indications of whether a particular mechanism of action of a specific drug has merit when taken into an *in vivo* model that recapitulates many aspects of the human disease, MS. EAE has been a remarkably valuable model, despite its many drawbacks.

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