Dissertation der Fakultät für Biologie der Ludwig-Maximilians-Universität München

Vaccination with DNA encoding a myelin autoantigen exacerbates experimental autoimmune encephalitis

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1. INDEX

2. SUMMARY

The ultimate goal in the treatment of autoimmune diseases is to reestablish tolerance to self antigens. One strategy to induce tolerance is to express the target autoantigen by DNA vaccination. In this work, the potential of vaccination with a DNA construct encoding the myelin oligodendrocyte glycoprotein (MOG), an important candidate autoantigen in multiple sclerosis, to induce tolerance and protect against experimental autoimmune encephalomyelitis (EAE) was assessed. Unexpectedly, mice vaccinated with MOG-DNA develop an exacerbated form of EAE when challenged with either MOG or an unrelated encephalitogen, myelin proteolipid protein. Disease exacerbation is due to the inability of DNA vaccination to tolerise the MOG specific T cell response and to the concomitant induction of a MOG-specific autoantibody response which is pathogenic, enhancing demyelination, inflammation and disease severity. These results suggest that tolerogenic strategies for autoimmune diseases based on DNA vaccination should be approached with caution.

3. INTRODUCTION

3.1. Experimental autoimmune encephalitis, a model for multiple sclerosis

3.1.1. Multiple sclerosis, an autoimmune disease?

Multiple sclerosis (MS) is a chronic disease of the central nervous system (CNS) characterised by plaques of focal destruction of myelin sheaths with relative preservation of axons and astroglial scarring (Lassmann et al., 1998). Although the most frequently affected sites are the optic nerves, the cervical portion of the spinal cord and the brainstem, the lesions can occur anywhere in the brain and spinal cord. Symptoms of multiple sclerosis vary widely depending on the localisation of the lesions, the most common symptoms being limb weakness, fatigue, cramps, parasthesiae, ataxia, bladder and bowel symptoms, visual symptoms (including diplopia) and vertigo. The clinical course of the disease is no less variable than the symptoms, but some patterns can be defined. In a high proportion of patients, the disease presents with episodes that recover. Further relapses occur randomly, with total or partial recovery (relapsing-remitting course). Eventually, this episodic symptomatology ceases in most patients and the course becomes slowly progressive (secondary progressive course). In 10 % of patients, however, disease is progressive from onset (primary progressive). Multiple sclerosis, which affects about one in a thousand individuals in the western world, has on average a duration of more than 25 years, and results in severe disability in a significant number of patients.

Although the etiology of multiple sclerosis is uncertain, family studies and studies of monozygotic and dizygotic twins indicate that genetic factors influence susceptibility to developing the disease. In particular, a strong association between the MHC class II allele HLA-DR2 and the relapsing form of the disease is described, suggesting an immunogenetic background (Martin et al., 1992). Indeed, it is widely believed that the disease is immune-mediated. This assumption is based on the histopathology of CNS lesions that show ongoing immune processes in the CNS of MS patients. The characteristic lesion in multiple sclerosis is the plaque, an area of circumscribed demyelination generally centred on a blood vessel in which relatively preserved axons are set in dense astroglial scar tissue. Loss of axons can however be significant and correlates well with persistent clinical deficit (Lassmann et al.,

1998). The demyelinating process is associated with inflammation both in the plaque and diffusely in the non-demyelinated CNS parenchyma. Inflammatory infiltrates are generally perivenular and consist mainly of macrophages and T lymphocytes, but B lymphocytes and antibody-producing plasma cells are also present (Esiri, 1977; Prineas and Wright, 1978; Traugott et al., 1983; Ozawa et al., 1994). Inflammation is associated with damage of the blood-brain barrier, an anatomical barrier that shields the CNS from the immune system (Katz et al., 1993). In active lesions, high numbers of macrophages that express activation markers and contain myelin degradation products in their cytoplasm are found (Ozawa et al., 1994; Bruck et al., 1995). In addition, upregulation of molecules that can potentiate an immune response such as MHC antigens and costimulatory molecules, as well as adhesion molecules and cytokines are found in active lesions (Traugott et al., 1983; Woodroofe and Cuzner, 1993; Washington et al., 1994; Cannella and Raine, 1995). Intrathecal IgG synthesis, demonstrated by the presence of oligoclonal bands in the cerebral spinal fluid on isoelectric focusing, is highly characteristic of multiple sclerosis and further argues in favour of an immune-mediated mechanism for the disease (Andersson et al., 1994). An intensive search for a possible infectious agent, implicating many different viruses over the years ranging from measles to the human herpes virus 6, has so far not identified a single causative agent, suggesting that multiple sclerosis may have an autoimmune etiology (Brody et al., 1972; Challoner et al., 1995). This hypothesis is supported by the presence of autoreactive T cells and autoantibodies specific for myelin antigens at a higher frequency in the peripheral blood and cerebrospinal fluid of multiple sclerosis patients compared to patients with other neurological diseases (Sun, 1993; Kerlero de Rosbo et al., 1993; Kerlero de Rosbo et al., 1997; Lindert et al., 1999; Kaye et al., 2000). Most striking, however, are the many clinical and pathological similarities between multiple sclerosis and experimental autoimmune encephalitis (EAE), an induced inflammatory disease of the CNS (Storch et al., 1998b).

3.1.2. Experimental autoimmune encephalitis

EAE shares many features with multiple sclerosis, such as acute, chronic and relapsing neurological dysfunction, including paralysis and ataxia, and a histopathology characterised by perivascular inflammation in the CNS. The disease can be induced in several different mammalian species by immunisation with either CNS tissue homogenate or individual myelin components. The first encephalitogen identified was the myelin basic protein (MBP), a major component of CNS myelin (Laatsch R.H. et al., 1962). Immunisation of rats with MBP in

complete Freund's adjuvant induces an acute ascending paralysis which first affects the tail and hind limbs, then the forelimbs, and generally resolves after a predictable period (Wekerle et al., 1994). Histologically, this disease is characterised by perivascular and parenchymal inflammation of the CNS, but demyelination is minimal. The demonstration that transfer of activated T cells specific for MBP was sufficient to induce EAE revealed the central role of $CD4^+$ T cells in pathogenesis and extensive studies subsequently characterised the mechanism of disease induction in this animal model (Paterson, 1960; Ben-Nun et al., 1981). The initial event is the activation of $CD4^+$ T cells outside the CNS by immunisation. Once activated, the T cells are able to cross the blood-brain barrier and enter the CNS irrespective of their antigen specificity, but only T cells specific for myelin antigens are stimulated by antigen-presenting cells within the CNS. This local interaction induces a full-blown inflammatory response, resulting in recruitment of mononuclear cells into the CNS and an increased permeability of the blood-brain barrier to serum proteins (Wekerle et al., 1994).

$CD4^+$ T cells can be grouped into functional subsets characterised by the cytokines they produce. In mice, EAE is mediated by inflammatory type 1-like helper $CD4^+$ T cells (Th1 cells) that produce IL-2 and IFNγ and promote the development of cellular immune responses such as cytotoxic lymphocytes and the production of the IgG2a immunoglobulin isotype. In contrast, Th2 cells that secrete the cytokines IL-4, IL-5, IL-6 and IL-10 are important in down-modulating inflammatory responses and regulate humoral immunity by promoting B cell activation and immunoglobulin isotype switching (Seder and Paul, 1994). The Th1 and Th2-mediated responses are frequently mutually exclusive and reciprocally regulated by their cytokine secretion.

Although immunisation with MBP results in clinical disease, it does not induce demyelination in either rats or primates (Martin et al., 1992), demonstrating that the Th1-mediated inflammatory infiltrates without demyelination are sufficient to induce clinical disease in these species. Early on, it was realised that MBP was not the only encephalitogen in the CNS and that immunisation with several other myelin proteins, including myelin proteolipid protein (PLP), myelin-associated glycoprotein (MAG), myelin oligodendrocytic basic protein (MOBP) and myelin oligodendrocyte glycoprotein (MOG), could result in EAE (Tuohy et al., 1988; Linington et al., 1993; Weerth et al., 1999; Holz et al., 2000). As increased autoimmune responses to all of these encephalitogens can be detected in multiple

sclerosis patients, they are all candidate autoantigens in multiple sclerosis (Sun, 1993; Kerlero de Rosbo et al., 1993; Reindl et al., 1999; Lindert et al., 1999; Kaye et al., 2000; Holz et al., 2000). MOG elicited particular interest as it is the only encephalitogen to induce severe demyelination in both rats and marmosets (Callithrix jacchus), a non-human primate, and thus to reproduce the complex pathology seen in multiple sclerosis (Genain et al., 1995; Adelmann et al., 1995).

3.1.3. The myelin oligodendrocyte glycoprotein

MOG is a 218 aminoacid, type I membrane glycoprotein that is present on the oligodendrocyte cell surface and on the myelin sheath produced by these cells (Brunner et al., 1989). MOG is encoded within the MHC complex (chromosome 6 in humans, 17 in mice), and the aminoacid sequence is highly conserved between species, with 89 % identity between rodents and humans (Gardinier and Matthieu, 1993; Pham-Dinh et al., 1993; Pham-Dinh et al., 1994). Sequence analysis revealed an Ig-like variable region domain, identifying MOG as a member of the immunoglobulin superfamily (Gardinier et al., 1992; Pham-Dinh et al., 1993). In addition, a single glycosylation site and two hydrophobic regions were found (Gardinier et al., 1992). The proposed membrane topology derived from analysis with domain-specific antisera is shown in Figure 1 (Kroepfl et al., 1996). The structure of MOG has led to the hypothesis that the protein may act as a receptor, but its function is still unknown. The generation of MOG knock-out mice has done little to solve the question, as these mice do not show a markedly altered phenotype (A. Dautigny, personal communication). In contrast to other myelin proteins such as MBP and PLP that are components of compact myelin, MOG is exposed on the outermost surface of the myelin sheath, where it is easily accessible to autoantibodies (Brunner et al., 1989). Furthermore, MOG is expressed exclusively in the CNS, whereas isoforms of MBP and PLP have been detected in the thymus and lymph nodes (Gardinier et al., 1992; Pribyl et al., 1996; Klein et al., 2000).

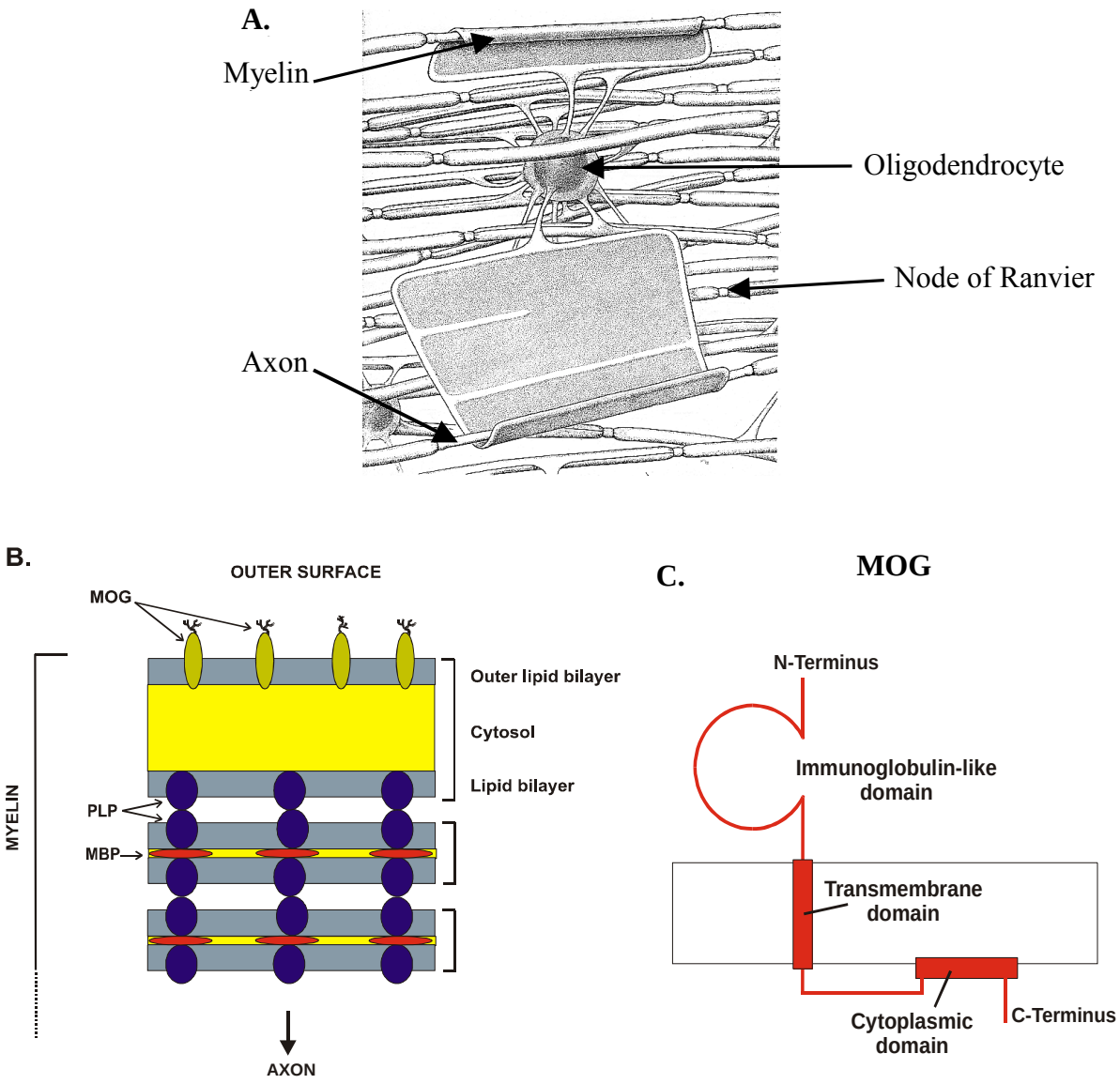

Figure 1: Topology of MOG in the myelin sheath

A) In the CNS, oligodendrocytes extend processes of their plasma membrane that wrap around axons to form the myelin sheath (here processes are shown partially unrolled) (from Morell and Norton, 1980).

B) Schematic cross-section of a CNS myelin sheath. Proteolipid protein (PLP) and myelin basic protein (MBP) are the main structural proteins of myelin. Through their interactions, they contribute to bring the membrane bilayers together and thus to the compaction of myelin. In contrast to MBP and PLP, MOG is localised on the outer surface of the myelin sheath.

C) Schematic representation of MOG. The N-terminal immunoglobulin domain is on the extracellular side of the membrane with a glycosylation site at position Asn 31. The transmembrane region extends from aminoacids 122 to 150, and a second, C-terminal hydrophobic region is thought to be associated with the cytoplasmic face of the membrane.

Although MOG is a minor component of myelin (0.01 - 0.05 % of all myelin protein) as opposed to MBP (20 %) and PLP (> 50 % of myelin protein), active immunisation with MOG induces a severe demyelinating disease that can be rapidly fatal (Johns et al., 1995; Genain et al., 1995). Demyelination is due at least in part to the action of pathogenic MOG-specific antibodies that bind MOG on the myelin surface and initiate complement-dependent and cell-mediated mechanisms. This was first proposed by Lebar et al. (1976), who showed that the demyelination observed in animals immunised with whole brain homogenate was due to complement-fixing antibodies specific for an autoantigen later shown to be identical to MOG (Lebar et al., 1986). Combined transfer experiments of encephalitogenic T cells and monoclonal antibodies in rats demonstrated that while T cells alone induce mainly inflammatory lesions in the CNS, the co-administration of MOG-specific antibodies produces widespread demyelination (Schluesener et al., 1987; Linington et al., 1988). In contrast, antibodies to MBP or PLP have no demyelinating effect (Seil et al., 1973; Seil and Agrawal, 1980). Further in vitro experiments reinforced the hypothesis that MOG-specific antibodies mediate demyelination by complement-dependent and cell-mediated mechanisms (Scolding and Compston, 1991; Piddlesden et al., 1993).

The role of B cells in MOG-induced EAE was further characterised by experiments in B cell deficient mice. Although these mice develop EAE following immunisation with an encephalitogenic MOG peptide, they are resistant to disease induced with whole MOG, indicating that MOG-induced EAE is mediated in part by B cells that may act as antigen-presenting cells (Lyons et al., 1999). The role of T cells should however not be underestimated, as there is an absolute requirement for encephalitogenic T cells in MOG-EAE. This is emphasised by the study of transgenic knock-in mice containing an immunoglobulin heavy chain specific for MOG. These mice have high numbers of MOG-specific B cells and anti-MOG antibodies (Litzenburger et al., 1998). Although these mice have an exacerbated clinical course following the transfer of encephalitogenic T cells, they do not develop spontaneous EAE. Thus, in contrast to other paradigms, MOG-EAE is a dual pathology model of disease mediated both by T and B cells, reproducing the immunological and pathological complexity of multiple sclerosis. The clinical relevance of MOG-induced EAE is further supported by the fact that MOG was identified as a potential target for autoimmune T and B cell responses in multiple sclerosis (Kerlero de Rosbo et al., 1993; Sun, 1993; Genain et al., 1999a; Lindert et al., 1999).

3.1.4. Tolerance induction in EAE

A major goal in the treatment of autoimmune diseases is the reestablishment of self-tolerance to the target autoantigen without compromising general immune responsiveness. Tolerance can be induced by three basic mechanisms: clonal deletion of autoreactive lymphocytes, functional inactivation of lymphocytes, and active suppression, mediated by regulatory T cells. T cell activation occurs upon interaction of naive T cells with antigen-presenting cells and requires both the binding of the antigenic peptide/MHC complex by the T cell receptor and a second, costimulatory signal provided by the interaction of the molecules B7.1 and B7.2 with CD28, their receptor on T cells. Generally, only professional antigen-presenting cells express costimulatory molecules, so that T cells that recognise self-antigens presented by tissue cells do not receive the essential costimulatory signal. This incomplete interaction leads to deletion of the T cells by programmed cell death (Miller and Basten, 1996) or to a state of non-responsiveness to antigen, termed anergy (Schwartz, 1996). In contrast, active suppression is mediated by $CD8^+$ suppressor T cells that secrete downregulatory cytokines such as TGFβ.

One strategy to enhance tolerance to tissue-specific antigens without the side-effects associated with long-term immunosuppression is the therapeutic administration of self-antigen in non-immunogenic form. Both the systemic administration of high dose soluble antigen and administration of antigen by mucosal (oral or intranasal) route have been shown to induce tolerance to the autoantigen and to protect against EAE. The intravenous or intraperitoneal injection of high doses of soluble MBP suppresses MBP-induced EAE both by anergy and deletion of autoreactive T cells (Gaur et al., 1992; Critchfield et al., 1994). It is probable that the high doses injected result in presentation of MBP by non-professional antigen-presenting cells, thus leading to tolerance. Following mucosal administration of MBP, low doses of antigen seem to favour tolerance by active suppression, whereas high doses of antigen promote T cell tolerance by anergy (Lider et al., 1989; Whitacre et al., 1991; Miller et al., 1992).

Until now, because of the emphasis on T cell-mediated models of EAE, treatments aiming to induce antigen-specific tolerance have been directed mainly at the T cell arm of the immune response. Indeed, in theory the elimination of autoreactive T cells should also silence the self-reactive B cell response which is dependent on the presence of activated T cells.

Activation of B cells occurs upon recognition of the appropriate peptide/MHC class II complex on B cells by functionally activated helper T cells. This interaction triggers the T cells to synthesize both CD40 ligand, a surface molecule that binds to CD40 on B cells, and the cytokines IL-4, IL-5 and IL-6. CD40 ligand and the released cytokines synergise to stimulate proliferation of B cells and their differentiation into antibody-secreting plasma cells. Therefore, provided that T cell tolerance to self-antigens is established, no T cell help will be available to activate self-reactive B cells. In addition, central and peripheral mechanisms exist to ensure B cell tolerance. Immature B cells that bind antigens in the bone marrow are either deleted or inactivated before completing maturation (Goodnow et al., 1988; Nemazee and Burki, 1989). In the periphery, mature B cells exposed to cell-surface antigens are deleted if they do not simultaneously receive the appropriate costimulatory signal from helper T cells (Russell et al., 1991). Furthermore, abundant soluble proteins can induce a state of anergy in mature B cells, characterised by reduced surface IgM and an inability to proliferate and differentiate after an immunogenic stimulus (Goodnow et al., 1989). Anergic B cells are then excluded from primary follicles in lymph nodes and spleen and are rapidly lost in the absence of T cell help (Cyster et al., 1994; Cyster and Goodnow, 1995).

The importance of targeting the B cell response to obtain antigen-induced tolerance was brought home by a report on systemic administration of MOG in MOG-EAE in the marmoset, a primate (Callithrix jacchus). As anticipated, repeated administration of high dose soluble MOG suppressed clinical signs of EAE during treatment (Genain et al., 1996). MOG-specific T cell proliferative responses were reduced and cytokine production was shifted from a Th1 to a Th2-type pattern. However, upon cessation of treatment a hyperacute, rapidly fatal form of disease emerged in the presence of high levels of autoantibodies. This study also highlights the transient character of antigen-induced tolerance. Indeed, the continuous presence of antigen is necessary to maintain a state of unresponsiveness generated by either clonal deletion or anergy (Liblau et al., 1997). An interesting alternative for generating antigen-specific tolerance is provided by DNA vaccination: this technique induces long-lasting expression of antigen at low levels following injection of naked DNA.

3.2. DNA vaccination

3.2.1. An introduction to DNA vaccination

DNA vaccination, the in vivo injection of plasmid DNA encoding an antigen of interest, represents a novel method of generating both humoral and cellular immune responses. Ten years ago, Wolff et al. first demonstrated direct gene transfer into mouse muscle in vivo. Using reporter genes, they showed that injection of plasmid DNA in a saline solution results in long-term in vivo expression of the encoded protein (Wolff et al., 1990). This was rapidly followed by the realisation that this procedure could elicit a specific immune response to the encoded antigen (Tang et al., 1992) and that this response could be protective in a number of preclinical models of viral, bacterial and parasitic diseases (Ulmer et al., 1993; Donnelly et al., 1995).

DNA vaccines consist of a bacterial plasmid containing a strong viral promoter, the cDNA of interest and a polyadenylation site. The plasmid is grown in bacteria, purified, dissolved in a saline solution and simply injected into the host. There the plasmid is taken up by host cells that produce the encoded protein, leading to an immune response against this antigen. Although many tissues can express the encoded antigen after DNA injection, expression is greatest following injection into muscle (Wolff et al., 1990). An alternative technique for DNA administration is the use of the gene gun. In this method, microscopic gold particles are coated with plasmid DNA and shot into the skin by the gene gun. The ballistically accelerated particles can thus penetrate cell membranes without killing the cells, resulting in transfection of cells in both the dermis and epidermis (Williams et al., 1991).

3.2.2. Immune responses generated by DNA vaccination

DNA vaccination has proven an effective method of inducing antibodies recognising a diverse array of proteins. Specific antibody responses have been generated by vaccination with DNA encoding viral, bacterial, parasitic and tumour antigens (Donnelly et al., 1997). Because DNA vaccines induce expression of antigen by host cells, they can result in production of protein with native conformation and posttranslational modifications that elicit antibodies of optimal specificity (Attanasio et al., 1997). In some cases, the antibodies have been shown to have a neutralising effect, thereby contributing to protection against challenge with the relevant infectious pathogen (Wang et al., 1993; Boyer et al., 1997; Lodmell et al., 1998).

DNA vaccination has also proven to be a valuable tool for generating monoclonal antibodies without the need for purified protein.

Although most conventional protein immunisations elicit specific antibody responses, cellular responses are generally weak or absent. In contrast, DNA vaccination readily induces MHC class I-restricted, cytotoxic T lymphocyte (CTL) responses (Ulmer et al., 1993). Cytotoxic lymphocytes generated by DNA vaccination are capable of recognising and killing target cells in vitro and mediate protection against challenge with a lethal dose of virus in vivo (Ulmer et al., 1993). CTL responses were found to persist for more than a year after DNA vaccination (Tighe et al., 1998). In addition, vaccination with DNA can modulate $CD4^+$ helper T cell responses. Although intramuscular DNA vaccination usually results in a Th1-like immune response, several factors including route of immunisation, cellular location of antigen and boosting can shift the developing immune response toward a Th2-like phenotype (Cardoso et al., 1996; Pertmer et al., 1996; Feltquate et al., 1997).

3.2.3. Mechanisms of antigen presentation

The nature of the antigen-presenting cells (APCs) responsible for the induction of the CTL response following DNA vaccination is of particular interest. The majority of $CD4^+$ T cells recognise peptides derived from exogenous proteins that are endocytosed by professional APCs and presented in the context of MHC class II molecules. Following DNA vaccination, professional APCs may therefore take up and present antigen produced by other transfected cells to induce a $CD4^+$ response. In contrast, $CD8^+$ CTLs generally recognise peptides derived from endogenous proteins presented in the context of MHC I molecules. In theory, induction of a CTL response by intramuscular DNA vaccination could therefore be via the following mechanisms: a) direct antigen presentation by transfected myocytes, b) transfection of professional APCs that produce and present the protein, and c) transfer of antigen from transfected myocytes to professional APCs, a phenomenon known as “cross-presentation” (Carbone et al., 1998). However, efficient stimulation of naive CTLs necessitates appropriate costimulation that may not be provided by transfected myocytes, and the low numbers of professional APCs in muscle make it unlikely that these APCs would be transfected following intramuscular DNA injection. Furthermore, several reports have provided evidence for transfer of antigen from myocytes to professional APCs as the main mechanism in induction of a CTL response. First, studies of bone marrow chimeras have shown that the principal

APCs involved in CTL priming following DNA vaccination are bone marrow-derived cells. In these experiments, irradiated mice of the H-2^{bxd} haplotype (the MHC locus in mice) received H-2^{b} or H-2^{d} bone marrow before DNA vaccination with the influenza nucleoprotein gene. The resulting CTL response was restricted to the donor haplotype, indicating that antigen presentation was performed by bone marrow-derived cells (Corr et al., 1996; Iwasaki et al., 1997). Second, transplantation of H-2^{k} myoblasts transfected with the influenza nucleoprotein gene into H-2^{kxd} mice induced a CTL response restricted to both the k and d haplotypes (Ulmer et al., 1996). This indicates that transfer of antigen from myocytes to professional APCs can occur and that transfection of professional APCs is not required for induction of a CTL response. Another study examined the CTL response in H-2^{b} or H-2^{d} mice with severe combined immunodeficiency (scid) that were DNA vaccinated intramuscularly several weeks before the transfer of splenocytes and bone marrow cells from H-2^{bxd} mice. In these mice a CTL response restricted to both H-2^{b} and H-2^{d} was detected, demonstrating that presentation of antigen was performed by the untransfected bone marrow-derived cells (Doe et al., 1996). These results indicate that transfer of antigen from myocytes to bone marrow-derived APCs can occur and is sufficient for class I MHC presentation of antigens after DNA vaccination.

In the case of DNA vaccination with the gene gun, it has been shown that dendritic cells (a type of professional APC) can be directly transfected in the skin by gold particles in vivo. The dendritic cells then migrate to the regional lymph nodes where they express the antigen. These cells can prime $CD8^{+}$ T cells in vitro without addition of antigen, indicating that directly transfected APCs can induce a CTL response following gene gun immunisation (Condon et al., 1996; Porgador et al., 1998).

3.2.4. Adjuvant effect of bacterial DNA

An important question raised by the efficacy of DNA vaccination is why the injection of DNA initiates such a potent immune response despite the production of minute amounts of antigen by transfected cells. One explanation has been offered by recent data showing that non-coding bacterial DNA has an adjuvant effect on the immune response. Bacterial DNA contains frequent immunostimulatory sequences (ISS) composed of an unmethylated CpG dinucleotide flanked by 2 purines on the 5' side and 2 pyrimidines on the 3' side. Since in mammalian DNA these sequences are uncommon and when present are usually methylated, unmethylated CpGs provide a recognisable pathogen-associated molecular pattern (PAMP),

that may allow the innate immune system to discriminate between pathogenic and non-pathogenic antigens (Janeway, 1998). These CpG dinucleotides elicit widespread effects on the immune system. They stimulate macrophages to produce IL-12, which in turn induces NK cells to produce IFNγ (Cowdery et al., 1996; Chace et al., 1997; Krieg et al., 1998). They induce maturation and activation of dendritic cells, and support CTL responses (Sparwasser et al., 1998; Tascon et al., 2000). CpGs also act on B cells that are activated, induced to proliferate and to produce immunoglobulins of the IgG2a isotype (Lipford et al., 1997; Yi et al., 1998). The cytokine milieu induced by ISS favours the generation of a Th1 biased immune response to the antigen encoded by the DNA vaccine, thereby acting as an adjuvant (Roman et al., 1997; Chu et al., 1997). Indeed, ISS are required for DNA vaccination to induce an efficient immune response (Sato et al., 1996).

3.2.5. Clinical applications of DNA vaccination

DNA vaccination has been found to be protective in a variety of models of infectious disease ranging from influenza and the human immunodeficiency virus (HIV) to listeriosis and tuberculosis (Ulmer et al., 1993; Boyer et al., 1997; Fensterle et al., 1999; Lowrie et al., 1999). Vaccination with DNA encoding tumour antigens has also been successfully used experimentally to protect against various tumours (Conry et al., 1995). In humans, DNA vaccines have raised immune responses against malaria, HIV-1 and metastatic melanoma in phase I and II clinical trials. A protective effect remains to be demonstrated in further long-term trials.

Interestingly, DNA vaccination has also proven effective in modulating the immune response in animal models of allergy. Vaccination with DNA encoding Der p 5, a house dust mite allergen, prevents IgE synthesis, histamine release and airway hyperresponsiveness in rats challenged with aerosolised allergen (Hsu et al., 1996). The main mechanism may be the production of Der p 5-specific $CD8^+$ T cells that inhibit the allergic response via the inhibitory effect of IFNγ on IgE-producing B cells and that affect the differentiation of Th2-like $CD4^+$ cells, which support IgE production. In addition, CpG oligodeoxynucleotides alone can prevent allergen-induced airway inflammation, presumably by shifting the Th2-type allergic response toward Th1 (Kline et al., 1998; Sur et al., 1999).

A protective effect of DNA vaccination has also been demonstrated in models of autoimmune diseases using several different strategies. One strategy is to generate an immune response targeting the effector mechanism of autoimmune disease. In a mouse model of MBP-EAE, DNA encoding a variable region gene of the T cell receptor expressed in the majority of pathogenic T cells suppressed disease (Waisman et al., 1996). Instead of depleting or blocking the relevant T cells bearing the targeted T cell receptor, DNA vaccination induced a shift from Th1 to Th2 in the cytokine pattern produced by the MBP-reactive T cells. The Th2 shift may thus have created a suppressive environment that blocks autoimmunity. In an other example, vaccination with DNA encoding the chemokines MIP-α and MCP-1, that are highly transcribed in the brain at the onset of disease, increased circulating antibody titers against these chemokines and prevented EAE in rats (Youssef et al., 1998). Another strategy using DNA vaccination to suppress immune-mediated disease consists of vaccinating with DNA encoding protective cytokines. For instance, DNA coding for IL-10 suppresses ongoing ocular inflammatory disease when administered topically (Daheshia et al., 1997). The disease, herpetic stromal keratitis, is mediated by inflammatory Th1 cells and the expression of Th2 cytokines, particularly IL-10, is involved in resolution of the inflammation.

Non-coding bacterial DNA itself can have an effect on autoimmune diseases. Boccaccio and colleagues demonstrated that vaccination with non-coding plasmid DNA decreases the incidence of MBP-EAE and the associated CNS inflammatory infiltrates (Boccaccio et al., 1999). Suppression of disease was also observed after vaccination with synthetic CpG oligodeoxynucleotides but not after injection of mammalian DNA. Interestingly, although IFNγ production by lymph node cells was increased, expression of this cytokine in the CNS was decreased, suggesting that suppression of EAE by non-coding bacterial DNA results from a regulatory function of IFNγ (Voorthuis et al., 1990).

Vaccination with DNA encoding self-antigens is another strategy to protect against autoimmune disease. In this case, the ectopic expression of self-antigen by DNA vaccination may enhance peripheral mechanisms of tolerance. However, expression of antigen in the immunostimulatory context of DNA vaccination might also be expected to generate immunity. Indeed, both scenarios have been demonstrated experimentally. DNA encoding either the MBP or PLP genes or their encephalitogenic peptides suppresses EAE compared to control plasmids (Ramshaw et al., 1997; Lobell et al., 1998; Ruiz et al., 1999). The

mechanism involved seems to be a decrease of Th1 responsiveness, as both T cell production of IL-2 and IFNγ and production of autoantigen-specific IgG2a were reduced after encephalitogenic challenge compared to animals vaccinated with control DNA (Lobell et al., 1998; Ruiz et al., 1999). In addition, a PLP-specific T cell line was unable to respond to CD28 costimulation in the presence of APCs loaded with DNA encoding the encephalitogenic PLP peptide (Ruiz et al., 1999). This suggests that DNA vaccination with self-antigen renders T cells anergic. However, in a conflicting report, DNA vaccination with the PLP gene and encephalitogenic epitopes in a similar construct actually enhanced PLP-induced EAE by increasing the number of PLP-specific encephalitogenic precursor cells (Tsunoda et al., 1998). The factors that influence the outcome of DNA vaccination with self-antigen therefore remain to be determined.

Until now, the role of DNA vaccination in suppressing EAE has been examined only in MBP and PLP-mediated EAE, which are purely T cell-mediated disease models. In contrast, both multiple sclerosis and MOG-EAE are mediated by a combination of T and B effector mechanisms, although an encephalitogenic Th1 T cell response is essential for the development of disease. In this situation, vaccination with MOG-encoding DNA could have various outcomes and it is not possible to predict the clinical effect on EAE. In particular, if T cell tolerance induced by DNA vaccination is incomplete the associated B cell response might even potentiate disease.

4. OBJECTIVES

The aim of this study was to assess the effect on EAE of vaccination with DNA encoding the myelin autoantigen MOG. As has been shown by vaccination with DNA encoding either MBP or PLP, the expression of myelin antigens in muscle following DNA vaccination can suppress EAE (Lobell et al., 1998; Ruiz et al., 1999). However, although EAE induced by immunisation with either of these autoantigens is frequently used as an animal model of multiple sclerosis, this disease is purely T cell-mediated (Martin et al., 1992). In contrast, the immunopathogenesis of multiple sclerosis is far more complex and involves both T and B cell responses to myelin autoantigens (Genain et al., 1999a). MOG-induced EAE is an animal model which also involves antibody-dependent immune effector mechanisms and therefore reproduces the immunological and pathological complexity of MS (Storch et al., 1998a; Weissert et al., 1998; Genain et al., 1999b).

Furthermore, unlike MBP and PLP that are expressed in lymph nodes and thymus, MOG is expressed exclusively in the CNS and is thus inaccessible to the immune system (Litzenburger, 1997; Pribyl et al., 1996; Klein et al., 2000). Tolerance to this antigen is therefore thought to be mediated by clonal ignorance, rather than by anergy or deletion. This is demonstrated by the presence in healthy individuals of autoreactive MOG-specific lymphocytes that, given the appropriate conditions, can be activated to induce an autoimmune response (Kerlero de Rosbo et al., 1993). Since expression of myelin antigens outside the CNS can lead to tolerance this work investigated the possibility of peripheral tolerance induction by MOG expression outside the CNS in the context of DNA vaccination. Practically, the effects of MOG-DNA vaccination on the antigen-specific T and B cell responses were to be analysed, as well as the effect on the clinical course of actively induced EAE.

5. MATERIALS AND METHODS

5.1. Materials

5.1.1. Chemicals

All chemicals not listed below were purchased from Merck (Darmstadt) when not otherwise indicated in the text.

Bovine serum albumine	Sigma, Munich
Concanavalin A	Sigma, Munich
SDS	Sigma, Munich
TEMED	Sigma, Munich
Tween 20	Sigma, Munich

5.1.2. Primer sequences

5SaMOG	ACGCGTCGACCTCAGCTTGGCCTGACCC
3BgMOG	CGAAGATCTGCTGGGCTCTCCTTCCGC
5pcDNA	TCAATGGGAGTTTGTTTTGGC
β-actin 1 (exon 3)	TGCTAGGAGCCAGGGCAGTAATC
β-actin 2 (exon 5)	TACAATGAGCTGCGTGTGGCC
MOG 1 (exon 2)	AGTTGGGGATGAAGCAGAGCTG
MOG 2 (exon 3)	TCGTAGGCACAAGTGCGATGAG

5.1.3. Peptides

MOG $^{35-55}$: MEVGWYRSPFSRVVHLYRNGK

MOG $^{93-107}$: KVTLRIQNVRFSDEG

PLP $^{139-154}$: HCLGKWLGHPDKFVGI

Peptides were synthesised by the Max-Planck Institute for Biochemistry in Martinsried.

5.1.4. Antibodies

8.18.C5: anti-MOG mouse monoclonal IgG1 antibody (Linington et al., 1984)

Alkaline phosphatase-conjugated antibodies:

(Southern Biotechnology Associates, USA)

goat anti-mouse Ig (H+L)

goat anti-mouse IgG

goat anti-mouse IgG1

goat anti-mouse IgG2a

goat anti-mouse IgG-FITC (Dianova)

5.1.5. Animals

SJL/J and C57/BL6 mice were purchased from Harlan or Charles River Wiga (Sulzfeld). 129/sv mice were obtained from the Max-Planck-Institute for Biochemistry, Martinsried. For all experiments, female mice aged 4-12 weeks were used (4-6 weeks for DNA vaccination). Animals were kept under specific pathogen-free conditions.

5.1.6. Frequently used buffers

PBS, pH 7.4

8.1 mM Na_2HPO_4

1.5 mM KH_2PO_4

2.7 mM KCl

137 mM NaCl

5.2. Molecular biology methods

5.2.1. Total RNA extraction from tissues

Total RNA was prepared by the single-step guanidine method according to manufacturer's instructions (Trizol, Gibco BRL). Tissues were flash-frozen in liquid nitrogen immediately after isolation and stored at –80°C until use. Frozen tissues were dropped into 1 ml Trizol in a 2 ml polypropylene tube and homogenized with a Polytron (Kinematica) homogenizer. After 5 min incubation at RT, extraction was performed with 200 µl chloroform (centrifuged 12000 g, 15 min at 4°C). The upper aqueous phase was taken and RNA precipitated with 0.5 ml isopropanol (10 min at RT). After centrifugation the pellet was washed in 75 % ethanol, dried and dissolved in 20-200 µl H_2O. RNA concentration was measured by spectrophotometer at A_{260}.

All RNA manipulations were performed with twice autoclaved pipet tips and tubes. All solutions were prepared with ultrafiltered and autoclaved H_2O.

5.2.2. Reverse transcription of RNA to cDNA

Reverse transcription of RNA to cDNA was performed according to manufacturer's instructions (Gibco BRL) in a total volume of 20 µl. For 1 µg total RNA 1 µl oligo $(dT)_{12-18}$ (500 µg/ml) and H_2O to a volume of 12 µl were added. Mixture was heated to 70°C for 10 min and chilled on ice. The following components were added:

4 µl 5x 1st strand buffer
2 µl 0.1 M DTT
1 µl 10 mM dNTP mix

After 2 min incubation at 42°C, 1 µl reverse transcriptase (Superscript II) was added and incubation was continued for 50 min. Reaction was stopped by heating at 70°C for 15 min. 2 µl were taken to perform a PCR reaction.

dNTP mix was purchased from MBI Fermentas. All other reagents used were from Gibco BRL.

5.2.3. Polymerase chain reaction (PCR)

The following reagents were added for a PCR reaction:

2 µl cDNA
5 µl 10x PCR buffer (Perkin-Elmer)
1 µl 10 mM dNTP mix
1 µl 5' primer (50-100 µM)
1 µl 3' primer (50-100 µM)
1.5 units Taq polymerase (Perkin-Elmer)
H_2O to a total volume of 50 µl

Samples were covered with 2 drops of mineral oil and amplified in a thermocycler (Perkin-Elmer) with the following conditions:

4 min at 94°C (denaturation), then 25-35 cycles with
1 min at 94°C (denaturation)
1 min at 60°C (annealing)
1 min at 72°C (elongation)

After the last cycle elongation was continued at 72°C for a further 10 min, then samples were cooled to 4°C and 8-10 µl were loaded onto an agarose gel for electrophoresis.

5.2.4. Separation of DNA fragments by agarose gel electrophoresis

Separation of DNA fragments was performed as described by Sambrook *et al*. (1989) on 0.8-1.2 % agarose gels with 0.5 µg/ml ethidium bromide in 1 x TBE at 5-10 V/cm. 0.2

volumes of loading mix were added to the DNA before loading on the gel. The fragments are separated by electrophoresis according to size and can be visualized in UV light due to the presence of ethidium bromide between the DNA strands. Ladder Mix (MBI Fermentas) was used as size standard.

DNA Loading Mix

100 mM EDTA pH 8
30 % glycerol
0.25 % bromophenol blue
0.25 % Xylene Cyanol

10 x TBE

89 mM Tris
89 mM H_3BO_3
2 mM EDTA

1 % TBE agarose gel

Boil in microwave 1 g agarose in 100 ml 1 x TBE, add 4 µl ethidium bromide (10 mg/ml, Sigma).

5.2.5. Southern blotting

The pattern of separated DNA fragments on an agarose gel can be transferred by capillary action onto a nylon membrane. Through hybridisation with homologous oligonucleotides the presence of specific sequences can be determined.

The agarose gel was washed for 15 min in 0.25 M HCl to depurinate and then for 30 min in transfer solution to denature DNA. On a glass plate were placed 2 sheets of 3MM paper so that the ends reached into a container filled with transfer solution. The gel, a prewetted Hybond + nylon membrane (Amersham) cut to size and 2 sheets of prewetted 3MM paper were laid successively on the plate. This was topped by a pile of absorbant paper and a weighted glass plate. The transfer was performed overnight at RT.

The DNA was then immobilised by UV crosslinking (1200 µJ, Stratalinker, Stratagene).

Transfer solution: 1.5 M NaCl, 0.25 M NaOH

5.2.6. 3'-end labeling of oligonucleotides with digoxigenin-11-ddUTP

The protocol from "DIG System user's guide for filter hybridization" (Boehringer Mannheim) was followed. The following reagents were incubated for 20 min at 37°C:

4 µl 5 x reaction buffer

4 µl $CoCl_2$ solution (5 mM)

100 pmol oligonucleotide (5 µM)

1 µl DIG-11-ddUTP (0.05 mM)

1 µl Terminal transferase 2.5 U/µl

The reaction was stopped by the addition of 1 µl 0.2 M EDTA, and the labeled oligonucleotides were precipitated with 2.5 µl LiCl and 75 µl ethanol (30 min at –70°C). The precipitate was collected by centrifugation at 13000 g for 5 min, washed with 70 % ethanol, dried and dissolved in 20 µl H_2O.

Reaction buffer, $CoCl_2$ solution, terminal transferase, DIG-11-ddUTP were purchased from Boehringer Mannheim.

5.2.7. Determination of DNA concentration

Concentration of DNA, RNA and oligonucleotides was determined by measuring the absorption at 260 and 280 nm by spectrophotometer (GeneQuant II, Pharmacia). At 260 nm with a 1 cm pathway, an optical density of 1.0 corresponds to 50 µg/ml of double-stranded DNA, 40 µg/ml of RNA and 34 µg/ml for oligonucleotides. The ratio of OD 260/OD 280 should be > 1.8, indicating purity of preparation.

5.2.8. Hybridisation of membrane-bound DNA to labeled oligonucleotides

The blotted membrane was preincubated for at least 2 h in hybridising solution at 44°C. The membrane was then transferred to a hybridising solution containing the digoxigenin-labeled oligonucleotide probe for at least 90 min at 44°C. The membrane was washed successively in:

2.5 x SSC, 0.1 % SDS (2 x 5 min at RT)

2.5 x SSC, 0.1 % SDS (2 x 5 min at 50°C)

Wash buffer (5 min at RT)

The membrane was then incubated for 30 min in freshly prepared anti-digoxigenin antibody solution and washed again 3 x for 10 min in wash buffer. The membrane was then washed in equilibration buffer for 5 min and covered for 5 min with chemiluminescence substrate (CDP-Star, Boehringer Mannheim) diluted 1/100 in equilibration buffer. Excess liquid was removed, the membrane was wrapped in clear plastic wrap and exposed to X-Ray film for 15 sec to 5 min at RT.

Buffer 1

100 mM maleic acid pH 7.5
150 mM NaCl

10 % blocking buffer

Dissolve blocking reagent (Boehringer Mannheim) in buffer 1, autoclave, store at 4°C

Hybridising solution

5 x SSC
1 % blocking buffer
0.1 % N-Lauroylsarcosine
0.02 % SDS
1µg/100 ml dig-labeled oligonucleotide

Wash buffer

100 mM maleic acid pH 7.5
150 mM NaCl
0.3 % Tween 20

Anti-digoxigenin antibody solution

19 ml buffer 1
1 ml 10 % blocking buffer
2 µl anti-digoxigenin alkaline phosphatase conjugate

Equilibration buffer

100 mM Tris-HCl pH 9.5
100 mM NaCl

10x SSC

3 M NaCl
0.3 M trisodium citrate

5.2.9. Isolation of DNA fragments from agarose gels

To isolate DNA fragments of different sizes after restriction, DNA was run on an agarose gel. The desired fragments were cut from the gel and DNA was isolated using a Jetsorb kit (Genomed) according to manufacturer's instructions.

5.2.10. Phenol-chloroform extraction and precipitation of DNA

To remove contaminants from DNA, an equal volume of a mixture of phenol, chloroform and isoamyl alcohol (proportions 25/24/1) was added to the DNA solution to be

cleaned. The mixture was centrifuged for 10 min at 13000 g to separate the phases. The aqueous upper phase was taken and DNA precipitated by the addition of 2 vol. 100 % ethanol and 0.1 vol. 3 M sodium acetate (15 min at –70°C). After centrifugation at 13000 g the pellet was washed in 70 % ethanol, dried and dissolved in H_2O. DNA concentration was measured by spectrophotometer.

Phenol-chloroform-isoamyl alcohol was from Gibco BRL.

5.2.11. Restriction of DNA

2-4 U of the desired restriction enzymes were added for 1 µg DNA in the appropriate restriction buffer. Volume of buffer used was 10 x the volume of enzyme added, as glycerol in the enzyme storage buffer may inhibit the reaction. The restriction mix was incubated 2 h at 37°C.

DNA restriction enzymes and buffers were purchased from Appligene (XhoI, SalI), Boehringer Mannheim (BglII) and MBI Fermentas (BamHI).

5.2.12. Dephosphorylation of DNA

To remove the 5' phosphate group from DNA fragments, 1 µl of shrimp alkaline phosphatase and 2 µl of 10 x dephosphorylation buffer were added directly to the restricted DNA in 20 µl of the restriction buffer. The mix was incubated for 1 h at 37°C and DNA was then cleaned by phenol-chloroform extraction and ethanol precipitation.

5.2.13. Ligation

DNA fragments with compatible ends were ligated with T4 DNA ligase. Amount of insert used was 3-6 times that of dephosphorylated vector. 1 U enzyme in 20 µl ligation buffer (Boehringer Mannheim) was added to 200 ng DNA and the reaction was incubated overnight at 16°C.

5.2.14. Generation of competent cells for electro-transformation

A fresh *E. coli* (DH5α) culture was prepared by inoculating 10 ml LB medium with a single colony and incubating overnight. 500 ml LB medium were inoculated with 5 ml (1/100) of the overnight culture and grown to a density of OD_{600} 0.5. The cell suspension was chilled on ice for 30 min and centrifuged at 4000 g for 15 min at 4°C. Cells were resuspended in 500 ml ice cold H_2O and centrifuged again. The pellet was resuspended in 250 ml ice cold H_2O, centrifuged and resuspended in 20 ml of ice cold 10 % glycerol. After another centrifugation

the pellet was resuspended in 2 ml of ice cold 10 % glycerol and stored in 40 µl aliquots at –70°C. The final transformation efficiency was approximately 10^7 cells/µg DNA (ideal efficiency: 10^9 transformants/µg). The whole procedure was performed in a cold room (8°C).

Luria-Bertoni (LB) medium

10 g tryptone (Difco)
5 g yeast extract (Difco)
10 g NaCl
5 ml NaOH 1M
add H_2O to a final volume of 1 l, autoclave

LB agar plates

15 g agar (Difco) in 1 l LB medium, autoclave

5.2.15. Transformation of bacterial cells by electroporation

40 µl of competent cells were thawed on ice, 1 µl DNA (5 ng) was added and left 1 min on ice. The cell suspension was then placed in a chilled sterile electroporation cuvette (0.2 cm) in a Gene Pulser (Bio-Rad) and pulsed once at 25 µF, 2.5 kV (Pulse Controller, Bio-Rad: 200 Ω). Cells were then immediately resuspended in 1 ml SOC medium. The cell suspension was incubated at 37°C for 1 h on a shaker in a polypropylene tube and then plated on LB-agar plates containing the appropriate selective antibiotics.

SOB medium

20 g tryptone
5 g yeast extract
0.5 g NaCl
2.5 ml KCl 1 M
H_2O to 1 l, pH 7
autoclave, add
10 ml $MgCl_2$ 1 M
10 ml $MgSO_4$ 1 M

SOC medium

add 1 ml sterile glucose 40 % to 9 ml SOB medium

5.2.16. Plasmid DNA preparation

To extract plasmid DNA from bacteria, cells are lysed with SDS and NaOH that denature DNA and proteins. The mixture is neutralized, causing the covalently closed plasmid

DNA to reanneal rapidly. While the chromosomal DNA and proteins precipitate, the plasmid DNA in the supernatant is concentrated by ethanol or isopropanol precipitation.

5.2.16.1 Minipreparation

A single bacterial colony was inoculated into 1 ml TB medium containing the appropriate antibiotic and incubated overnight in a shaker at 37°C. 50 µl were taken, added to 50 µl glycerol and stored at –20°C. The remaining culture was then processed according to the manufacturer's instructions (Jetquick, Genomed). The bacterial pellet was resuspended in 210 µl of solution 1, cells were lysed by the addition of 210 µl of solution 2 and incubated at RT for 5 min. The suspension was neutralized with 280 µl of solution 3 and centrifuged 10 min. at 13000 g. The supernatant was loaded onto a Jetquick column and centrifuged for 1 min. The column was washed with 700 µl of solution 4 and centrifuged twice for 1 min. The plasmid was then eluted by adding 50 µl H_2O onto the column and centrifuging for 2 min. Yield was approximately 20 µg.

Terrific broth (TB) medium

12 g tryptone
24 g yeast extract
4 ml glycerol
Add H_2O to 900 ml, autoclave.
Add 100 ml sterile salt solution (0.17 M KH_2PO_4; 0.72 M K_2HPO_4)

Solution 1

50 mM Tris-HCl (pH 8)
10 mM EDTA
100 µg/ml RNase A

Solution 2

200 mM NaOH
1 % SDS (w/v)

Solution 3

3 M potassium acetate, pH 5.5
guanidine hydrochloride

Solution 4

ethanol, NaCl, EDTA, Tris/HCl

5.2.16.2 Mega- and Gigapreparations

For preparing large amounts of plasmid DNA for vaccination, Mega- and Gigapreparations (Qiagen) were performed. Maximum yield is 2.5 mg (Megaprep) and 10 mg (Gigaprep). A starter culture was generated by inoculating a single bacterial colony from a selective plate into 10 ml LB medium containing 100 µg/ml ampicillin and incubating for 8 h in a shaker (300 rpm) at 37°C. The starter culture was diluted 1/500 into selective LB medium (500 ml for the Megaprep, 2.5 l for the Gigaprep) and the culture was grown for 12-16 h at 37°C until the cell density measured by photometer was 1.2 at OD_{600}. Cells were harvested by centrifugation for 30 min at 5000 rpm at 4°C. Isolation of plasmid DNA was performed using a Qiafilter kit (Qiagen) according to manufacurer's instructions. Quality of DNA obtained was verified by spectrophotometer and by agarose gel after restriction.

5.2.17. DNA sequencing

Sequencing of DNA was performed by Toplab in Martinsried.

5.3. Cell biology methods

5.3.1. Culture media

Eagle's Hepes solution (EH)

500 ml DMEM (Dulbecco's modification of Eagle's medium, 4500 mg/ml glucose, without sodium pyruvate, Gibco BRL)
5 ml Penicillin-Streptomycin (10^4 IU/ml penicillin, 10 mg/ml streptomycin, Gibco BRL, 1 %)
12.5 ml HEPES-buffer 1 M (Gibco BRL), pH 7

Complete DMEM

500 ml DMEM (4500 mg/ml glucose, Glutamax I, with sodium pyruvate, Gibco BRL)
5 ml Penicillin-Streptamycin (10^4 IU/ml penicillin, 10 mg/ml streptomycin, Gibco BRL, 1 %)
5 ml non-essential aminoacids (Gibco BRL, 1 %)
5 x 10^{-5} M mercaptoethanol (5 ml of a 10 µl/10 ml EH, Sigma)

Inactivation of fetal calf serum (FCS)

FCS (Gibco BRL) was incubated in a water bath at 56°C for 30-45 min and stored in aliquots at –20°C.

Ag8 culture medium

Complete DMEM
10 % inactivated FCS

T cell primary culture medium

Complete DMEM

1 % mouse serum

Freezing medium

45 % culture medium or EH

45 % FCS

10 % DMSO to prevent formation of crystals

Concanavalin A (stock solution)

Concanavaline A (Con A, Pharmacia) 1 mg/ml in PBS

^{3}H-thymidine (stock solution)

^{3}H-thymidine (Amersham) 50 µCi/ml in EH

All manipulations were performed with sterile reagents under a laminar flow hood.

5.3.2. Culture of the myeloma cell line X63-Ag8.6.5.3 (Ag8)

The myeloma cell line Ag8 was grown in Ag8 culture medium at 37°C and 10 % CO_2. Cells were diluted 1/3 in fresh medium every 3-5 days.

5.3.3. Determination of cell number

Trypan Blue staining differentiates between live and dead cells, as the membrane of dead cells but not of live cells is permeable to the staining. The cell suspension was mixed with Trypan Blue at the appropriate dilution (1/2 – 1/100) and 10 µl were transferred to a Neubauer hemocytometer. 100-200 live cells (unstained, round and bright) were counted under the microscope. Cell number was calculated as follows:

$$[\text{Cells in one big square}] \times [\text{dilution factor}] \times 10^4 = \text{Cells/ml}$$

5.3.4. Electro-transformation of Ag8 cells

The application of a high intensity electric field creates temporary pores in the cell membrane allowing DNA to enter the cell. 2 x 10^7 Ag8 cells were resuspended in 700 µl PBS and transferred to a chilled sterile 0.4 mm electroporation cuvette (Bio-Rad). The cells were pulsed twice in a Gene Pulser (Bio-Rad) at 0.28 kV, 500 µF with a time constant of 4.1 sec (Pulse Controller, Bio-Rad: 200 Ω). Cells were then resuspended in 50 ml culture medium and plated on two 48-well plates (500 µl/well). Control wells containing untransfected cells were included.

5.3.5. Selection of transfected Ag8 cells (Ag8/MOG)

To select transfected cells, the neomycin resistance gene was included in the expression vector (pcDNA) used for transfection. This gene confers resistance against geneticin (G418). 24 h after electroporation, cells were placed in DMEM culture medium containing 1.5 mg/ml G418. Medium was changed every 2-3 days for 14 days. At this time no live cells remained in the control wells. MOG expression was verified by Western blotting and wells with the highest expression were expanded and frozen in aliquots in freezing medium (24 h at –20°C, 24 h at -70°C, then storage in liquid nitrogen).

5.3.6. Flow cytometry

Cell surface antigens can be recognized by specific antibodies coupled to fluorescent markers and detected by flow cytometry. A fluorescence-activated cell sorter (FACS) measures the light emission generated by single cells flowing through a laser beam (excitation wavelength 488 nm). Several parameters can be measured: size (forward scatter, FSC) and granularity (side scatter, SSC) of cells, as well as fluorescence emitted from surface-bound antibodies coupled to fluorochromes. The following fluorochromes were used: fluorescein isocyanate (FITC, emission at 530 nm, FL1) and phycoerythrin (PE, emission at 578 nm, FL2). Dead cells were stained with propidium iodide as the membrane of live cells is impermeable to this compound.

10^5-10^6 cells from a single cell suspension were added in 50 µl FACS buffer per well of a 96-well plate. Cells were washed twice by centrifugation (1200 rpm, 6 min) in 100 µl FACS buffer and resuspended in 50 µl FACS buffer containing an optimal concentration of the first antibody (unlabeled or coupled to biotin or to FITC) or mouse serum to be tested. Cells were incubated on ice for 20-45 min and washed twice with FACS buffer. For a second staining the pellet was resuspended in 50 µl FACS buffer containing another antibody (PE). To detect an unlabeled first antibody a second FITC-labeled antibody directed against the Ig of the first antibody species was added. To detect biotin in the first staining PE-coupled streptavidin (Amersham) was added. Cells were again incubated on ice for 20-45 min, washed twice and transferred to FACS tubes in 100-200 µl FACS buffer. Propidium iodide was added at a concentration of 1 µg/ml. Fluorescence was measured with a FACS Scan (Becton-Dickinson) and results were analysed with Becton-Dickinson software (Lysis).

FACS buffer

1 x PBS; 3 % FCS, 0.1 % NaN_3

5.3.7. Sorting of Ag8/MOG cells by FACS

To select Ag8/MOG cells expressing the highest amount of MOG, cells were sorted by FACS. The FACS Sorter (Becton-Dickinson) was sterilized by filling all tubes with 70 % ethanol for at least 1 h. Ethanol was washed away with PBS/0.2 % glucose. Cells to be sorted were stained with a monoclonal antibody specific for MOG (8.18.C5) and streptavidin-PE and resuspended at a density of 10^6 cells/ml in 3 ml PBS/0.2 % glucose. Propidium iodide was added at a concentration of 1 µg/ml. FACS parameters were set to accept only live and highly MOG-positive cells (top 10 %), and sorting was performed into tubes coated with PBS/BSA. Sorted cells were resuspended in Ag8 culture medium with 1.5 mg/ml G418 and grown for at least 1 week before verifying MOG expression by FACS.

5.3.8. Complement-dependent cytotoxicity assay

Antibodies bound to the cell surface can induce cell lysis in the presence of complement. To assay lysis efficiency of serum antibodies from DNA-vaccinated mice, 10^5 Ag8 or Ag8/MOG cells/well were incubated with serial dilutions of test serum or specific antibody in 100 µl Ag8 culture medium for 20 min on ice. Complement was added to a final dilution of 1/80 and cells were incubated 45 minutes at 37°C with 10 % CO_2. The tetrazolium salt WST-1 (Boehringer Mannheim), a colorimetric indicator of metabolic activity in cells, was added at a dilution of 1/10, and cells were incubated further at 37°C with 10% CO_2. The absorbance at 450 nm was read on a MR-4000-ELISA-Reader (Dynatech) at different time points.

Complement used was reconstituted rabbit complement (Behring) and was stored at –20°C in single-use aliquots for a maximum of 3 weeks.

5.3.9. Isolation of blood cells for flow cytometry

After section of the tail vein 0.5 – 1 cm from the distal end 200-400 µl of blood were collected in a tube containing 70 µl heparin (10 u/µl, Sigma), mixed immediately and kept on ice. To induce hypoosmolar lysis of erythrocytes, 1 ml ACK buffer was added and mixture was incubated for 5 min on ice. After centrifugation (5 min at 1200 rpm, 4°C) the pellet was resuspended in ACK buffer, again incubated and centrifuged. Cells were washed once in FACS buffer and staining was performed.

ACK buffer

4.3 g NH_4Cl (0.15 M)
0.5 g $KHCO_3$ (1 mM)
18.6 mg Na_2 EDTA (Titriplex III, 0.1 mM)
in 500 ml H_2O, pH 7.2-7.4, sterile filtered

5.3.10. Preparation of a single cell suspension from mouse tissues

Popliteal lymph nodes and/or spleen were isolated from mice on day 28 after DNA vaccination or on day 10 after conventional immunisation and immediately placed in EH medium on ice. Organs in EH were forced through a wire mesh screen (Sigma) and suspension was passed several times through a 23G canula to generate a single cell suspension. Cells were washed once in EH medium and resuspended in culture medium or FACS buffer.

5.3.11. Primary culture of T lymphocytes

Lymph node cells or splenocytes in single cell suspension were incubated in primary culture medium for 2-3 days at 37°C with 10 % CO_2 at a density of 5 x 10^6 cells/ml. For cytokine ELISA cells were incubated at a density of 2.5 x 10^6 cells in 1 ml primary culture medium. Supernatant was taken at 48 h and stored at –70°C.

5.3.12. T cell specificity assay

In this assay, T lymphocytes are incubated in the presence of various concentrations of antigen. Labeled thymidine is incorporated into the newly synthesized DNA of dividing lymphocytes, thus giving a measure of antigen-specific proliferation of T lymphocytes. 2 x 10^5 lymph node cells or 10^6 splenocytes in 200 µl of primary culture medium were added per well in a 96-well plate in the presence of specific antigen (MOG, PLP), unspecific antigen (purified protein derivative or PPD, from mycobacteria), the mitogen Con A or without antigen. Cells were incubated for 2 days at 37°C with 10 % CO_2. 20 µl of ^{3}H-thymidine stock solution were added (final concentration 1 µCi/well) and cells were incubated for a further 16-18 h. Cells were then transferred to a fiberglass filter, washed and specific activity was measured in a beta-counter (Matrix 96 Direct Beta Counter, Packard).

5.4. Biochemical methods

5.4.1. SDS-PAGE

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970) with the Mini-Protean II system (Bio-Rad) using 1 mm spacers. Electrophoresis was performed in Laemmli buffer at 150 V for 1 to 2 h. All samples were mixed with 0.2 vol. 5 x loading buffer and incubated at 95°C for 5 min before loading. Molecular weight markers used were prestained and unstained broad range (Bio-Rad)

12.5 % acrylamide separation gel (for 10 ml)

4.2 ml acrylamide
2.5 ml Lower Tris
3.5 ml H_2O
10 µl TEMED (Tetramethylethylenediamine)
9 µl APS 40 % (ammonium persulfate)

Stacking gel (for 5 ml)

0.75 ml acrylamide
1.25 ml Upper Tris
3.0 ml H_2O
5 µl TEMED
12.5 µl APS 40 %

Laemmli buffer (for 1 l)

3 g Tris [25 mM]
14.3 g Glycin [190 mM]
1 g SDS [0.1 %]

Lower Tris (for 100 ml)

18.2 g Tris [1.5 M]
4 ml 10 % SDS solution [0.4 %]
pH 8.8

Upper Tris (for 100 ml)

50 ml Tris.HCl 1 M pH 6.8 [500 mM]
4 ml 10 % SDS solution [0.4 %]

Loading buffer (for 100 ml)

15.6 ml Tris.HCl 2 M pH 6.8 [312 mM]
10 g SDS [10 %]
57.5 ml 87 % glycerin [50 %]'

25 ml β-mercaptoethanol [25 %]

0.5 g bromophenolblue [0.5 %]

5.4.2. Staining of proteins in polyacrylamide gel

To reveal separated proteins in polyacrylamide gels, gels were stained with Coomassie Blue staining solution for 30 min, then incubated for 30 min – 1 h in destaining solution.

Coomassie Blue staining solution (for 1 l)

1 g Coomassie Brilliant Blue R (Bio-Rad) [0.1 %]

500 ml methanol [50 %]

75 ml acetic acid [7.5 %]

Destaining solution (for 1 l)

200 ml methanol [20 %]

75 ml acetic acid [7.5 %]

5.4.3. Western blotting

5.4.3.1 Electrotransfer of protein

The electrotransfer of protein from a polyacrylamide gel onto a nitrocellulose membrane (ECL, Amersham) was performed according to Tobwin et al. (1979). After separation of proteins by SDS-PAGE, the gel was incubated for 5 min in blotting buffer and placed in a transfer chamber (Bio-Rad) against a nitrocellulose membrane (Hybond ECL, Amersham). Electrotransfer was performed for 1 h at 100 V and 4°C. To verify successful transfer of protein and note the position of the marker, the membrane was stained for 5-15 min in 0.2 % Ponceau solution (Sigma) and destained with H_2O.

Blotting buffer (for 1 l)

3 g Tris [25 mM]

14.3 g glycin [190 mM]

1 g SDS [0.1 %]

200 ml methanol [20 %]

5.4.3.2 Detection of membrane-bound protein by chemoluminescence

To detect the presence of membrane-bound protein with specific antibodies, unspecific binding sites on the membrane were first blocked with blocking buffer for 1 h at RT. The membrane was then incubated with the unlabeled specific antibody at the appropriate concentration for 1 h at RT. After washing 3 x in TBST, the membrane was incubated in the

appropriate concentration of the second peroxidase-conjugated antibody for 1 h. The membrane was again washed 3 x and detection by chemiluminescence was performed according to manufacturer's instructions (ECL, Amersham).

Tris buffer solution – Tween (TBST) 10 x (for 1 l)

12.1 g Tris [100 mM]
87 g NaCl [1.5 M]
5 ml Tween 20 [0.5 %]

Blocking buffer

5 % milk powder in 1 x TBST

Antibody solution

2 % milk powder in 1 x TBST

5.4.4. Determination of protein concentration according to Lowry

In this assay, the complexing of soluble protein with an alkaline cupric tartrate reagent forms a purple –blue color in the presence of phenol. The assay was performed according to manufacturer's instructions (Sigma) with different dilutions of protein solution and absorption was measured at 600 nm. A calibration curve was determined from standard protein samples and concentration of the test sample was calculated from the absorption value.

5.4.5. Purification of recombinant MOG from bacteria

The expression of recombinant MOG was performed in transformed E. coli bacteria of the DH5α strain. The expression vector introduced (pQE12, Qiagen) contains an IPTG inducible promoter, the cDNA coding for the extracellular domain of rat MOG (aminoacids 1-125) and a histidine tag sequence coding for 6 histidines at the 3' end, to allow purification by nickel-chelate chromatography (Amor et al., 1994).

5.4.5.1 Expression of recombinant protein in bacteria

400 ml of selective culture medium were inoculated with a transformed E. coli glycerol stock and incubated overnight at 37°C on a shaker. After centrifugation at 5000 rpm for 20 min the bacterial pellet was resuspended in 4 x 1 l of selective culture medium without glucose and shaken at 37°C. When the absorption at 600 nm reached 0.7, 10 ml of 0.1 M IPTG (BioTech Trade) were added per liter of culture to induce MOG expression. Incubation was continued for a further 3-4 h, bacteria were centrifuged (20 min, 5000 rpm, 4°C), resuspended in 200 ml PBS and centrifuged again. The pellet was stored overnight at –20°C.

Selective culture medium

LB medium; 50 µg/ml ampicillin; 25 µg/ml kanamycin; 0.1 % glucose (w/v)

5.4.5.2 Lysis of bacteria

The pooled bacterial pellet was resuspended in 40 ml PBS and sonicated for 40 min on ice (Branson Sonifier 450) to lyse the cells. Recombinant MOG is insoluble in PBS and forms inclusion bodies. After centrifugation (4°C, 16000 rpm in SS34 rotor, 30 min) the pellet containing the MOG inclusion bodies and other insoluble proteins was resuspended in urea buffer and sonicated for 40 min to dissolve the inclusion bodies. The suspension was again centrifuged and the supernatant containing the dissolved MOG was used for affinity chromatography.

Urea buffer

6 M urea; 0.5 M NaCl; 0.1 M KH_2PO_4; pH 8

5.4.5.3 Nickel-Chelate Affinity Chromotography

The column (Chelating Sepharose Fast Flow, Pharmacia) was washed sequentially with dH_2O and with 1 % EDTA/0.05 % Tween 20 to remove the 70 % ethanol used for storage and any remaining nickel. The column was loaded with a 1 % nickel (Ni^{2+}) solution, washed with dH_2O and equilibrated with urea buffer. The column was then loaded with the sample at a rate of 0.5 ml/min and washed with 200 ml wash buffer. The elution was performed with an linear imidazole gradient with 200 ml elution buffer. Fractions of 4 ml were collected and analysed by SDS-PAGE. The protein concentration in the eluate was monitored by photometer at 280 nm. The column was regenerated with 1 % EDTA, 0.05 % Tween 20 to remove all nickel and then with H_2O.

Wash buffer

40 mM imidazole in urea buffer

Elution buffer

500 mM imidazole in urea buffer

5.4.5.4 Dialysis

After SDS-PAGE analysis the protein-containing eluate fractions were pooled and placed in a dialysis tube (exclusion 3500 Da, Biomol). Dialysis was performed twice

overnight at 4°C against 5 l acetate buffer. Protein concentration was determined and purity of the MOG preparation was verified by SDS-PAGE.

Acetate buffer

20 mM Na acetate; 20 mM acetic acid; pH 3

5.4.6. Enzyme-linked immunosorbent assay (ELISA)

With this technique (Engvall and Perlmann, 1971) the binding of antibodies to a plate-bound antigen or antibody can be measured by an enzymatic reaction. Serum antibody titers can thus be determined.

A 96-well ELISA plate (polyvinyl coating, Costar) was coated overnight at 4°C with 10 µg/ml antibody or antigen in coating buffer (100 µl/well). The plate was washed 3 x with wash buffer (ELISA-washer, Nunc). Unspecific binding sites on the plate were blocked with 200 µl/well block buffer for at least 1 h at 37°C or overnight at 4°C. The plate was washed again and 50-100 µl of the serum to be tested were added to each well at appropriate dilutions (for example 1/100) in coating buffer. The plate was incubated at least 1 h at 37°C and washed. To detect the adsorbed antibody an anti-mouse antibody coupled to alkaline phosphatase was added at a dilution of 1:1000 in coating buffer and incubated at least 1 h at 37°C. Plate was washed 3 x. To develop a colored reaction p-nitrophenyl phosphate (Sigma) was used as substrate for alcaline phosphatase. 100 µl substrate buffer were added in each well and reaction was developed for 5 to 30 min. The plate was read at 405 nm in a spectrophotometer (MR 4000 ELISA Reader, Dynatech) at different time points.

Coating buffer: 0.02 % Na azide in PBS

Block buffer: 1 % (w/v) BSA (Sigma); 0.1 % Na azide in PBS

Wash buffer: 0.05 % Tween 20; 0.02 % Na azide in PBS

Substrate buffer: 1 M diethanolamine (Sigma); 0.02% Na azide; 4 mM $MgCl_2$; pH 9.8. Store in the dark.

5.4.7. Cytokine ELISA

The measurement of cytokines in T lymphocyte culture supernatant was also performed by ELISA. In this assay cytokines were detected by sandwich ELISA: plates were coated with an anti-cytokine antibody, and bound cytokines were revealed by a second, biotinylated anti-cytokine antibody followed by streptavidin coupled to peroxidase. Assays were performed according to manufacturer's instructions (kits from R & D Systems and

Endogen). A calibration curve was determined for standard concentrations and titer of the sample was calculated from the absorption value at 450 nm.

5.5. Animal experimentation

5.5.1. Preparation of serum

300-500 µl of blood were collected in 1.5 ml polypropylene tubes by section of the tail vein approximately 0.5 cm from the distal end of the tail. Blood was left to coagulate a few hours at RT or overnight at 4°C. It was then centrifuged 10 min at 1200 rpm, supernatant was taken and centrifuged again. This step was repeated until the supernatant was clear. The serum obtained was stored at –20°C.

5.5.2. DNA vaccination of mice

For DNA vaccination the plasmid DNA was injected intramuscularly into the tibialis anterior muscle. As this muscle is only 4-5 mm in diameter in the mouse, a tubing was inserted over the 27G canula, covering all but 1-2 mm of the tip, to prevent too deep insertion of the needle. The injection technique was practiced by injecting India ink into the muscle after anesthesia, then sacrificing the animal and assessing presence of the ink in the correct muscle.

For DNA vaccination female mice (age 4-12 weeks) were anesthesized with ether and injected in each tibialis anterior muscle with 50 µg DNA in PBS (1 mg/ml). MOG-specific antibody titers in serum were verified by ELISA 4 weeks after immunisation.

5.5.3. Conventional immunisation of mice with Freund's adjuvant

The antigen solution was mixed with an equal volume of complete or incomplete Freund's adjuvant. The mixture was forced through a 24G tube (Hamilton) fixed between 2 syringes until a hard emulsion was obtained. Mice were injected subcutaneously in footpads, tail base and flanks after ether anesthesia with 100 –200 µg of antigen in 100-200 µl of emulsion. To enhance the immunisation 300 ng of pertussis toxin in PBS (1 µg/ml) were injected intraperitoneally on the day of immunisation and again 48 h later.

Incomplete Freund's adjuvant (IFA) was purchased from Gibco BRL. Complete Freund's adjuvant (CFA) was prepared by adding 10 mg/ml mycobacteria tuberculosis (H37RA, Difco) to IFA and grinding with mortar and pestle.

5.5.4. Clinical evaluation of EAE

After immunisation with encephalitogens, mice were regularly weighed and clinically assessed. The clinical signs of EAE were evaluated according to the following scale:

0: no signs of disease

1: loss of muscle tone of tail

2: paresia of hind limbs

3: paralysis of one or both hind limbs

4: paralysis of one or both hind limbs and paresia of fore limbs

5: moribund or dead animal

Half-points were also given.

In accordance with federal guidelines animals with a score of 4.5 for 2 consecutive days or reaching a score of 5 were sacrificed. These mice were then scored as 4.5 or 5, respectively, for the remainder of the experiment (mean cumulative clinical score).

5.5.5. Perfusion of mice

For histopathological examination mice were perfused with paraformaldehyde (PFA) solution. Under ether narcosis mice were thoracotomised and the upper vena cava was sectioned. 10-20 ml of PFA solution were injected slowly into the left ventricule. Brain and vertebral column were removed, postfixed overnight in PFA solution, then stored in PBS at 4°C.

PFA solution (freshly prepared): 4 % PFA in PBS was solubilised at 60°C with the addition of a few drops of NaOH and stirred for 1 h.

5.6. Histological methods

5.6.1. Cryosections

5.6.1.1 Freezing of tissues for cryosections

Freshly isolated spleen, lymph nodes, brain and spinal cord were immediately placed in ice-cold PBS. Organs were then placed on wet Whatman paper, covered with embedding medium and quickly frozen on dry ice. Brain was divided transversally in 3 parts (forebrain, midbrain and cerebellum) before freezing. To minimize freezing artefacts muscle tissue was frozen in a beaker of isoamyl alcohol placed in liquid nitrogen and embedded just prior to cutting. Organs were stored at –70°C.

5.6.1.2 Preparation of glass slides

To prevent detachment of tissues, glass slides were coated with a 1 % gelatin solution and air-dried before use.

Gelatin solution: 1 % gelatin in H_2O, dissolve by heating to 65°C for 30 min.

5.6.1.3 Cutting of sections

10 µm sections were cut by cryostat (Mod. 2700-Frigocut, Reichert-Jung) and stored at –70°C.

5.6.2. Hematoxylin-Eosin (HE) staining

This staining reveals the general tissue structure. Cytoplasm is stained red (eosin) and nuclei blue-violet (hematoxylin).

Sections on glass slides were dried at RT for 30 min. Fixation was performed in –20°C acetone for 2 min and sections were again dried for 2 min. Sections were placed in a hematoxylin solution (Dako) for 10 min and then in lukewarm tap water (10 min). The slightly alkaline pH changes the hematoxylin staining from red to blue-violet. Slides were washed in ultrafiltered H and stained in an 0.01 % aqueous solution of eosin for 5-10 sec. To dehydrate, sections were placed in a series of increasing ethanol concentrations:

2 x 70 % ethanol (short)
1 x 90 % ethanol (short)
1 x 96 % ethanol (3 min)
2 x 100 % ethanol (10 min)
2 x Rotihistol (Roth, 10 min)

Slides were mounted with an ethanol-based mounting medium (Entellan, Merck) and dried at RT.

5.6.3. Luxol-Fast-Blue (Klüver-Barrera) myelin staining

This technique stains myelin bright blue. The tissue section is counterstained with calcium red (stains nuclei) to show the general structure. The slides were incubated 2 h at 56 °C in Luxol-Fast-Blue solution, then washed in 70 % ethanol and H_2O. Selective destaining of non-myelin tissues (differentiation) was achieved by a short dip in a 0.05 % lithium carbonate solution. Slides were washed in 70 % ethanol and H_2O. The differentiation was repeated until desired intensity was obtained. Sections were then counterstained for 6 min with calcium red,

dehydrated in a series of increasing ethanol concentrations and Rotihistol and mounted with Entellan (Merck).

Luxol-Fast-Blue

1 g Luxol-Fast-Blue
1 l 96% ethanol
5 ml 10 % acetic acid

Calcium red

1 g calcium red
1 l 5 % aluminium sulfate
Heat to dissolve, filter.

5.6.4. Immunohistochemistry

This technique was used to specifically detect T cells, B cells and macrophages or MOG in tissue sections.

Sections on glass slides were dried at RT for 30 min. Fixation was performed in –20°C acetone for 2 min and slides were again dried for 2 min. Sections were circled with Dako Pen to prevent spreading of the solutions pipetted. After equilibrating for 5 min in TBS, unspecific sites were blocked with 20 % serum in TBS for 20 min. Slides were washed 3 x in TBS and the biotinylated specific antibody was pipetted onto the section at a concentration of 1/50 – 1/200 in TBS with 4 % BSA and 1 % xenogeneic serum. Negative controls were covered with TBS/BSA/serum solution. After 1-2 h incubation, slides were washed 3 x in TBS, incubated with streptavidin-alkaline phosphatase complex (Dako) for 45 min and washed again. The development was performed with freshly prepared neofuchsin solution (Dako) containing levamisole, an inhibitor of endogenous alkaline phosphatases. Neofuchsin turns bright pink in the presence of alkaline phosphatase. Development was stopped after 1-3 min with H_2O. Counterstaining was performed with hematoxylin and slides were mounted in a water-based medium (Glycergel, Dako).

Alternatively, staining was performed with streptavidin-coupled horseradish peroxidase (1/1000, Pharmingen) and developed with 3,3'-diaminobenzidin (DAB, Sigma). DAB turns brown in the presence of horseradish peroxidase.

1x TBS (Tris buffer solution)

0.15 M NaCl
0.05 M Tris-HCl
pH 7.6

5.7. Statistical analysis

Error bars represent standard error of the mean (SEM), calculated as follows:

$$SEM = \frac{SD}{\sqrt{n-1}}$$

SD: standard deviation

Tests of significance used were Student's t-test for T cell proliferation and cytokine production (comparison of independent values from 2 sample groups with normal distribution) and Mann and Whitney's U-test for clinical scores (comparison of independent values from 2 sample groups, not normal distribution).

6. RESULTS

6.1. Protocol of immunisation with MOG

Protein immunisation against MOG was performed using the recombinant Ig-like extracellular domain of MOG (MOG^{Igd}, see Figure 1A). The induction of EAE with MOG^{Igd} includes many experimental parameters that can modulate the clinical course of the disease. A preliminary series of experiments was conducted to determine the optimal conditions in MOG-EAE for the issues addressed in this work. As the first question was to examine possible tolerance induction by MOG-DNA vaccination, experimental conditions were selected to obtain early and severe clinical disease. For this reason the highly susceptible SJL mouse strain was chosen for initial experiments and a high dose of MOG^{Igd} (200 μg) in complete Freund's adjuvant was used for immunisation. The effect of an intraperitoneal injection of pertussis toxin (PTX) as adjuvant on the day of immunisation and again 48 h later was assessed in Figure 2. Results shows that injection of MOG/CFA simultaneously in flanks, tail base and footpads without addition of PTX induces a disease with very late onset (day 25) and a maximum score of only 1.5. In contrast, both protocols of immunisation that include PTX induce much earlier and more severe disease. It was concluded that PTX as adjuvant was necessary to induce strong clinical disease.

The necessity of injection of MOG/CFA in the footpads was also evaluated, as the resulting local inflammation may cause discomfort to the mice. After immunisation in flanks, tail base and footpads and using PTX, EAE onset occured on day 10 and disease score rapidly reached a peak of over 3 points. Disease was lethal in 3 of 5 mice between day 13 and day 28. When immunisation was performed only in flanks and tail base, but not in the footpads (also with PTX), disease onset was 2 days later and reached an initial peak of only 1.5. Mean maximum score then slowly increased to reach 3 points on day 35. Disease was lethal in 2 of 5 mice late in the course of disease (days 25 and 31). It was concluded that injection of MOG/CFA in the footpads was also necessary to induce early and severe EAE.

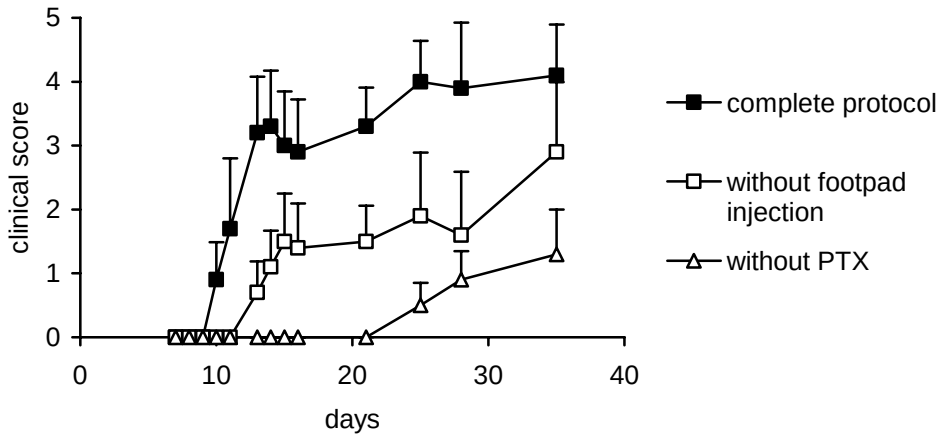

Figure 2: Immunisation protocols for MOG-EAE
EAE was induced in SJL mice by immunisation with 200 µg MOG ^{Igd} in CFA. One group of mice received the 200 µg MOG/CFA s.c. distributed in tail base, flanks and footpads and additionally PTX i.p. on day of injection and again 48 h later (■). Another group was treated following the same protocol but without injection in footpads (□). The 3rd group of mice received the same treatment (including footpad injection) but no PTX (Δ). Data represent mean cumulative clinical score of 5 mice/group.

6.2. Expression of MOG following DNA vaccination

6.2.1. Construction of vectors for DNA vaccination

Two different expression vectors were generated for MOG-DNA vaccination. First, mouse MOG cDNA was amplified by PCR from a cDNA preparation derived from SJL mouse brain. The primers used for PCR (5SaMOG and 3BgMOG) were specific for the 5' and 3' untranslated regions of the MOG cDNA and included each a restriction site to facilitate cloning. The PCR product of 830 bp containing the complete MOG coding sequence including the signal sequence was then inserted into two different expression vectors, pHSE3' and pcDNA3.1(-) (Figure 3). The first vector, pHSE 3', contains the mouse H2-K (MHC-I) promoter, ensuring expression of the insert in nearly all types of mouse cells, and includes a β-globin splice site and polyadenylation site and an Ig enhancer. The second plasmid, pcDNA 3.1 (-) (Invitrogen), contains a strong human CMV promoter and enhancer that directs high expression in most cell types, and the bovine growth hormone polyadenylation site. In addition, it contains the neomycin resistance gene which allows positive selection for expression in mammalian cells. Correct insertion of MOG cDNA in both vectors was verified by sequencing.

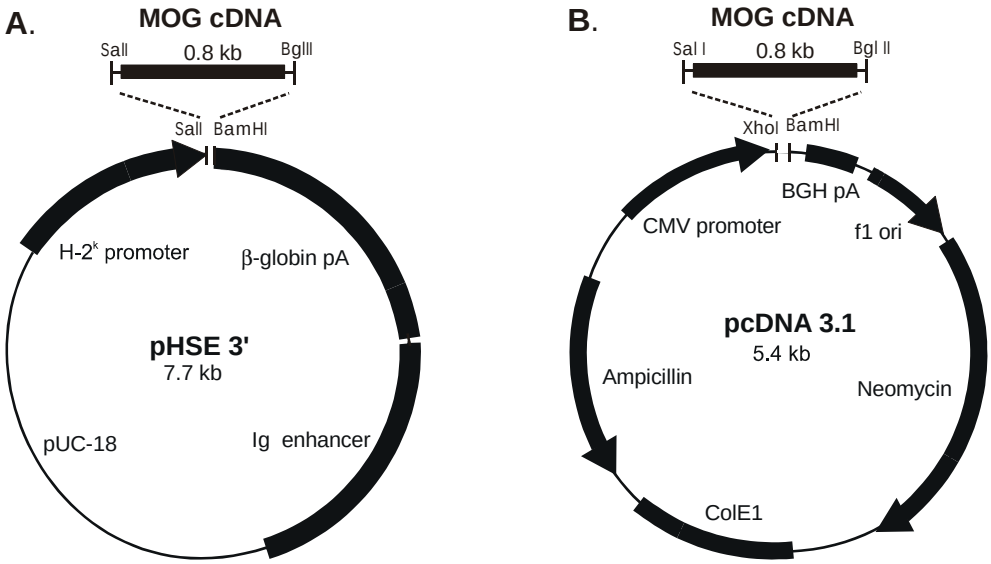

Figure 3: Maps of pHSE/MOG and pcDNA/MOG
The complete coding sequence of MOG cDNA including the signal sequence was cloned via SalI and BglII restriction sites into the expression vectors (A) pHSE 3' and (B) pcDNA 3.1 (+) (Invitrogen). The cDNA is placed under the control of the H-2^k promoter and the CMV promoter, respectively.

6.2.2. Expression of pcDNA/MOG in a transfected cell line

The accuracy of MOG expression induced by pcDNA/MOG was analysed in the mouse myeloma cell line X63-Ag8.6.5.3 (Ag8, (Kearney et al., 1979)). This was performed with pcDNA/MOG as the vector contains the neomycin resistance gene allowing positive selection with G418 in mammalian cells. Ag8 cells were stably transfected with pcDNA/MOG under constant positive selective pressure with G418 and protein expression was assessed by Western blotting and FACS analysis. Protein extracts from SJL mouse brain, MOG-transfected (Ag8/MOG) and untransfected (Ag8) cells, and recombinant MOG were separated by SDS-PAGE, transferred to a nitrocellulose membrane and detected with 8.18.C5, a monoclonal antibody specific for MOG (Figure 4). Natural glycosylated MOG extracted from CNS myelin (brain) consists of 218 aminoacids and runs at a molecular weight of 28 kDa (Gardinier et al., 1992). Brain extract from MOG knock-out mice (brain KO, a kind gift from A. Dautigny) was used as negative control and demonstrated the absence of the 28 kDa band in these mice. Expression of MOG was detected in Ag8/MOG cells but not in Ag8 cells. The protein was similar in size to brain MOG, although it ran slightly slower on the gel. This may reflect a different pattern of glycosylation of MOG by the Ag8 cell line. Recombinant rat and human MOG^{Igd} were used as additional positive controls. These recombinant proteins

consist solely of the extracellular Ig-like domain (aminoacids 1-125 for rat MOG, and 1-120 for human MOG, (Pham-Dinh et al., 1993)) and have a molecular weight of approximately 17 respectively 19 kDa, but frequently dimerise to give a band at approximately 34 kDa (Gardinier et al., 1992).

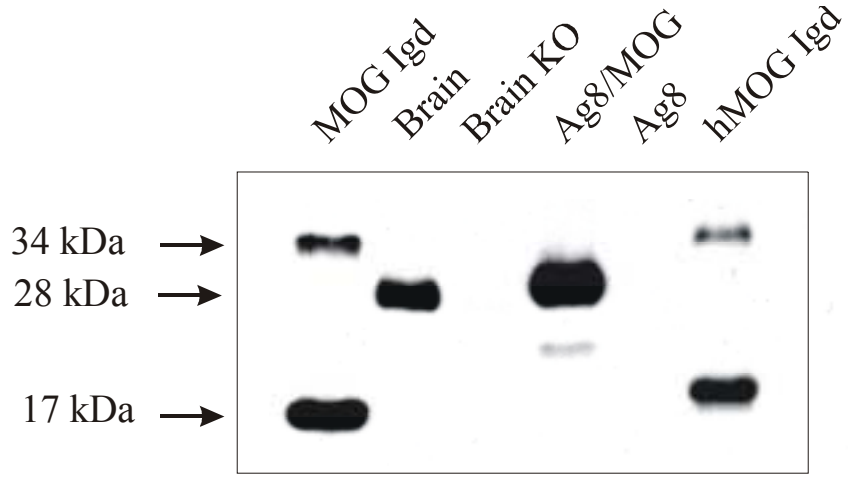

Figure 4: Western blot detection of MOG expression in Ag8/MOG cells
Brain and cell culture protein extracts and the recombinant Ig-like domains of rat and human MOG were separated on a 12.5 % SDS-polyacrylamide gel, transferred to a nitrocellulose membrane and detection was performed with 5 µg/ml 8.18.C5, a monoclonal antibody specific for MOG.
$\underline{MOG^{Igd}}$: recombinant rat MOG (Ig-like domain) (10 µg); $\underline{hMOG^{Igd}}$: recombinant human MOG (Ig-like domain) (25 µg); Brain KO: Protein extract from the brain of MOG knock-out mice; Ag8/MOG: MOG-transfected myeloma cells; Ag8: untransfected cells.

These results demonstrate that the pcDNA/MOG expression vector is indeed functional in mammalian cells. However, as MOG is a membrane protein and the construct included the MOG signal sequence, it was expected that the protein should be expressed on the cell surface. This was verified by analysing Ag8/MOG cells by flow cytometry for surface MOG expression. Staining with the 8.18.C5 monoclonal antibody detected MOG expression on the surface of more than 95 % of Ag8/MOG transfectants but not on untransfected cells (Figure 5), indicating that transfection with pcDNA/MOG results in the integration of MOG into the membrane and its translocation to the cell surface.

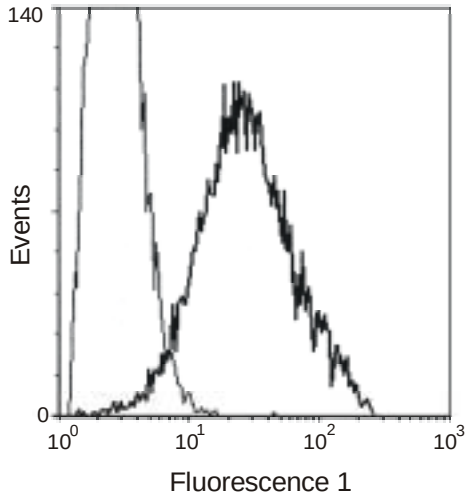

Figure 5: FACS analysis of surface MOG expression in Ag8/MOG transfectants
Ag8/MOG transfectants (bold line) and untransfected Ag8 cells (thin line) were surface stained with the MOG-specific 8.18.C5 antibody (5 µg/ml) and analysed by flow cytometry to detect the presence of MOG on the cell surface.
Fluorescence 1: detection of the Cy2-labeled second antibody that binds to 8.18.C5.

6.2.3. Expression of pcDNA/MOG *in vivo*

Having shown that transfection with pcDNA/MOG induces the expression of MOG *in vitro*, *in vivo* expression following intramuscular injection of pcDNA/MOG in SJL mice was examined by Western blotting and immunohistochemistry. However, unlike the situation *in vitro*, it was not possible to demonstrate the presence of MOG protein in muscle following DNA vaccination. Protein extracts of injected muscles of mice vaccinated with 50 µg pcDNA/MOG in each tibialis anterior muscle were assessed by Western blotting with 8.18.C5 at days 0, 2, 7 and 14 after DNA injection. The presence of a MOG band could not be detected in any of the examined muscles (data not shown). Similarly, immunohistochemical analysis using biotinylated 8.18.C5 antibody for detection on muscle sections spanning the entire tibialis anterior in 100 µm intervals could not positively identify the presence of MOG protein at any time point after vaccination (data not shown). It is however not unusual for the encoded protein to be expressed at levels below detection following DNA vaccination (see introduction).

The more sensitive technique of RT-PCR was then used to demonstrate transcription of MOG cDNA *in vivo* following vaccination with pcDNA/MOG. Two pairs of primers were used for amplification of cDNA derived from injected tibialis anterior muscle: the first pair (5SaMOG and 3BgMOG) is specific for the 5' and 3' untranslated regions of the MOG cDNA, thereby detecting both MOG transcripts and the MOG cDNA-containing plasmid. The

second pair (5pcDNA and 3BgMOG) contains one primer specific for the 3' untranslated region of the MOG cDNA and one for the promoter region of the expression vector, thereby detecting exclusively the presence of the pcDNA/MOG vector.

It has been reported that MOG is specifically expressed in the CNS of mice and rats, but not in the peripheral nervous system or in lymphoid organs (Gardinier et al., 1992; Litzenburger, 1997). The present experiment extends these observations and demonstrates that MOG is not expressed in adult mouse striated muscle (Figure 6, day 0). However, although MOG could be detected by RT-PCR in injected muscle following DNA vaccination the results were very variable. In total 15 mice in 3 different experiments were analysed. MOG transcripts or MOG cDNA were in many cases not detectable. However, in some animals it was possible to demonstrate persistance of the pcDNA/MOG plasmid in the muscle for up to 14 days after injection (Figure 6).

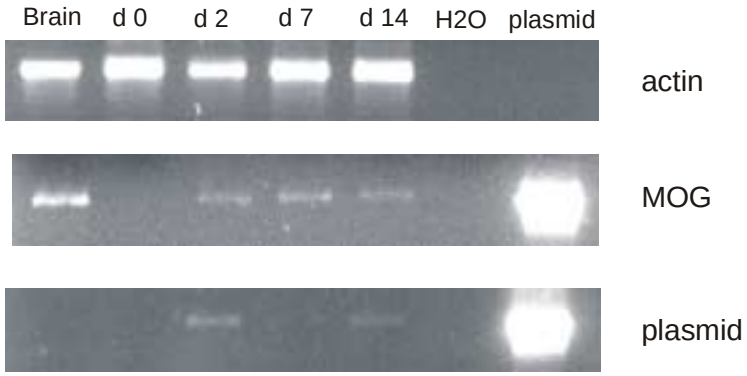

Figure 6: Persistance of pcDNA/MOG in mouse muscle after injection
RT-PCR from total RNA isolated from mouse brain and muscle before DNA injection (day 0) and from injected muscle 2, 7 and 14 days after pcDNA/MOG vaccination. 2 µl of cDNA or 30 ng of plasmid DNA (pcDNA/MOG) were used as template for PCR amplification (30 cycles) with primers specific for β-actin (exons 3 and 5, product 700 bp), MOG mRNA and/or cDNA (5' and 3' UTR, product 830 bp) and pcDNA/MOG (promoter region in the vector and 3' UTR of the MOG cDNA, product 1060 bp).

In other animals the presence of MOG transcripts was visible from 2 to 14 days after pcDNA/MOG injection in the absence of detectable plasmid DNA (Figure 7A). Hybridisation of the amplified fragments with a specific oligonucleotide (mMOG2) confirmed that the amplified products contained the MOG coding sequence (Figure 7B). That the band indeed represented MOG mRNA and not plasmid DNA was confirmed by performing RT-PCR

without reverse transcriptase (Figure 7C). No trace of MOG mRNA or plasmid DNA was identified in spleen, thymus or liver at any time point (data not shown).

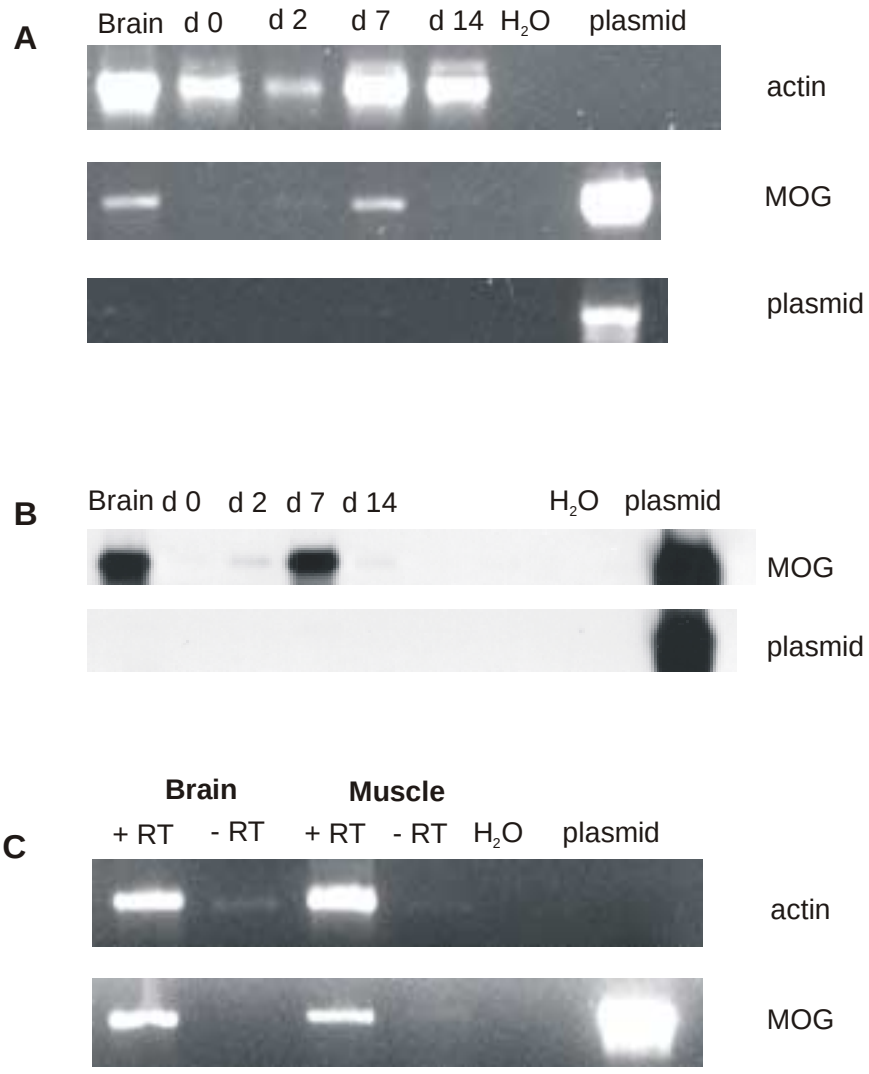

Figure 7: RT-PCR analysis of MOG expression in muscle following DNA vaccination
A) RT-PCR from total RNA isolated from mouse brain and muscle before DNA injection (day 0) and from muscle 2, 7 and 14 days after vaccination. Primer pairs used were described in Figure 6. 30 ng pcDNA/MOG were used as positive control. B) DNA from gel shown in A was transferred to a membrane and hybridised with a MOG-specific oligonucleotide. C) RT-PCR performed for total RNA from brain (day 0) and muscle (day 7) in the presence and absence of reverse transcriptase (RT).

In view of the variability demonstrated in the persistence of pcDNA/MOG and its transcription in muscle, the high sensitivity afforded by immune assays was then used to assess the effect of vaccination with MOG-DNA.

6.3. Antibody response induced by DNA vaccination

The effect of intramuscular vaccination with MOG-DNA on the immune response was first examined by assessing the MOG-specific antibody response in SJL mice following DNA vaccination with either pHSE/MOG or pcDNA/MOG. As SJL mice are highly susceptible to MOG-induced EAE (Litzenburger, 1997), this strain was most likely to show an immune response to MOG following DNA vaccination. ELISA of serum obtained from mice vaccinated with pHSE/MOG identified an autoantibody response to MOG^{Igd} 4 weeks after vaccination that was absent in control pHSE treated animals (Figure 8A). In mice vaccinated with pcDNA/MOG, MOG-specific IgG antibodies were detected from two weeks p.v., reached a plateau 1 to 2 weeks later and were still present up to 5 months later (Figure 8B). In control pcDNA vaccinated mice, MOG-specific antibody titers remained at background levels comparable to those of naive animals. DNA vaccination with both pHSE/MOG and pcDNA/MOG generated comparable titers of MOG-specific antibodies at 4 weeks following DNA vaccination, indicating that both expression vectors can efficiently induce a productive immune response in SJL mice. However, as pcDNA is a standard vector for DNA vaccination and to allow comparison with other studies, pcDNA/MOG was chosen to be used in all further experiments.

Of 77 SJL mice vaccinated with pcDNA/MOG, only 4 showed no increase in the MOG-specific antibody titer at 4 weeks after vaccination, indicating a response rate of nearly 95 %. This demonstrates a much higher efficiency of MOG-DNA vaccination than was detected by RT-PCR, so that measurement of anti-MOG antibody titers proved to be a reliable readout for the efficacy of MOG-DNA vaccination. In all further experiments, all mice were tested for the presence of anti-MOG IgG antibodies at 4 weeks after DNA vaccination and non-responders were excluded from the study.

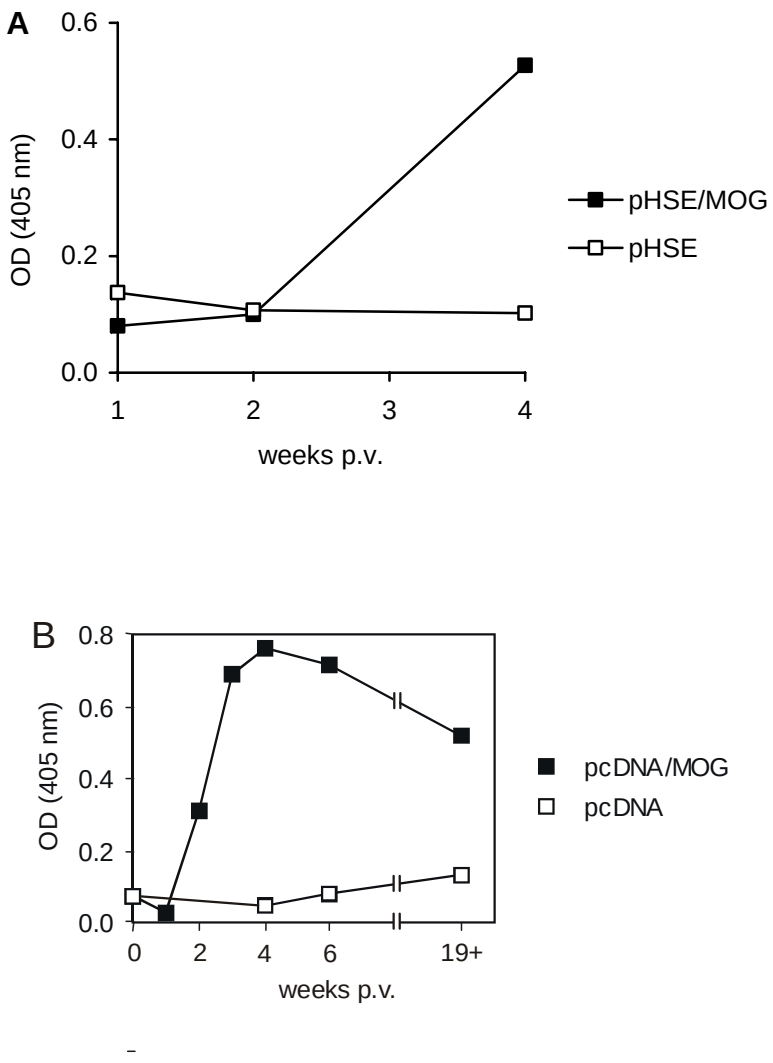

Figure 8: MOG-specific antibody response induced by DNA vaccination
Time course of the MOG-specific Ig response following vaccination with DNA determined by ELISA. Sera were pooled from ≥3 mice and pipetted at a dilution of 1/100 on a MOG-coated ELISA plate. A) MOG-specific total Ig after vaccination with pHSE/MOG or pHSE. B) MOG-specific IgG after vaccination with pcDNA/MOG or pcDNA. Point 19+: serum pooled from 2 mice each from 19 and 23 weeks p.v. Assay was performed in triplicate.

Intramuscular DNA vaccination with other constructs encoding viral or bacterial proteins has been reported to induce an immune response skewed towards the production of antibodies of the IgG2a isotype, suggesting an inflammatory Th1-like response (see introduction). To see if this was also the case for MOG-DNA vaccination, the relative amounts of MOG-specific IgG1 and IgG2a in serum of MOG-DNA vaccinated SJL mice were determined by ELISA 4 weeks after vaccination, at the peak of MOG-specific IgG titers. For

comparison, sera from SJL mice immunised with MOG^{Igd} in CFA were analysed at 28 days p.i.. Conventional immunisation with MOG^{Igd} in CFA induced high anti-MOG antibody titers of both the IgG1 and IgG2a isotypes (Figure 9). In contrast, the response following pcDNA/MOG vaccination was clearly dominated by antibodies of the IgG2a isotype (Figure 9) in all groups of mice examined. Elevated titers of MOG-specific IgG1 antibodies were detected in only one experiment concomitantly with IgG2a antibodies. The IgG2a antibody titer was much higher in MOG ^{Igd}-immunised mice than in MOG-DNA vaccinated animals, with an O.D. following conventional immunisation that was 4 to 5 times higher than in DNA vaccinated mice. Control mice vaccinated with the pcDNA vector showed no MOG-specific antibodies of either IgG1 or IgG2a isotypes (Figure 9), as expected from the absence of anti-MOG IgG antibodies.

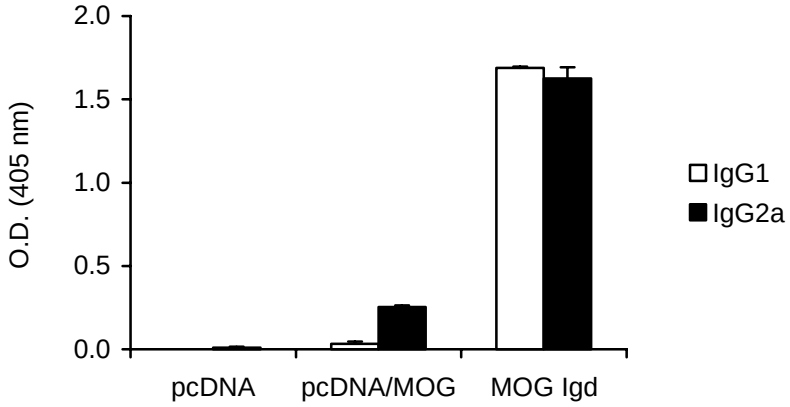

Figure 9: IgG1 and IgG2a immune response following DNA vaccination
MOG-specific IgG1 and IgG2a serum titers were assayed by ELISA in mice 28 days after vaccination with pcDNA or pcDNA/MOG or after conventional immunisation with MOG^{Igd} in Freund's adjuvant. Sera were pooled from ≥3 mice and assay was performed in triplicate. Data are representative of at least 2 independent experiments.

The predominance of MOG-specific IgG2a autoantibodies following MOG-DNA vaccination suggests the induction of a Th1-like autoimmune response to MOG. However, the MOG-DNA vaccinated mice did not develop any clinical or histopathological signs of MOG-EAE for several months after DNA vaccination, indicating that the Th1-like response generated by MOG-DNA vaccination is not sufficient to induce disease.

6.4. T cell response

The original aim of this study was to induce tolerance to a CNS self-antigen as described in other EAE models. The isotype analysis of the antibody response following

MOG-DNA vaccination indicated a productive Th1 associated response, but paradoxically no induction of clinical EAE. This could reflect the generation of a subclinical population of MOG-specific Th1 T cells. This was examined in more detail by investigating MOG^{Igd}-induced proliferation and cytokine production by splenocytes from MOG-DNA vaccinated mice. Splenocytes were isolated from mice vaccinated with either pcDNA or pcDNA/MOG 5 weeks after vaccination and cultured for 3 days in the presence of MOG^{Igd}. However, the splenocytes showed no MOG-specific proliferation (Figure 10A) and neither MOG-induced IFNγ nor IL-4 production was detected by ELISA in the cell culture supernatant (data not shown). The absence of a measurable T cell response despite the presence of a productive antibody response indicates that the frequency of MOG-specific lymphocytes in the spleen following DNA vaccination was below detection levels.

The T cell response was then examined in lymph node cells taken from mice immunised with MOG^{Igd} 4 weeks after pretreatment with either pcDNA or pcDNA/MOG. As expected following MOG^{Igd} immunisation, dose-dependent MOG-specific proliferation of cells from both pcDNA and pcDNA/MOG pretreated animals was observed. However, no evidence for the suppression of T cell reactivity by MOG-DNA vaccination was found. The proliferative response to MOG^{Igd} was unaffected or even slightly enhanced in 1 of 3 independent experiments using lymph node cells prepared from pcDNA/MOG treated mice (Figure 10B). In no case was the proliferative response suppressed relative to control plasmid treated mice.

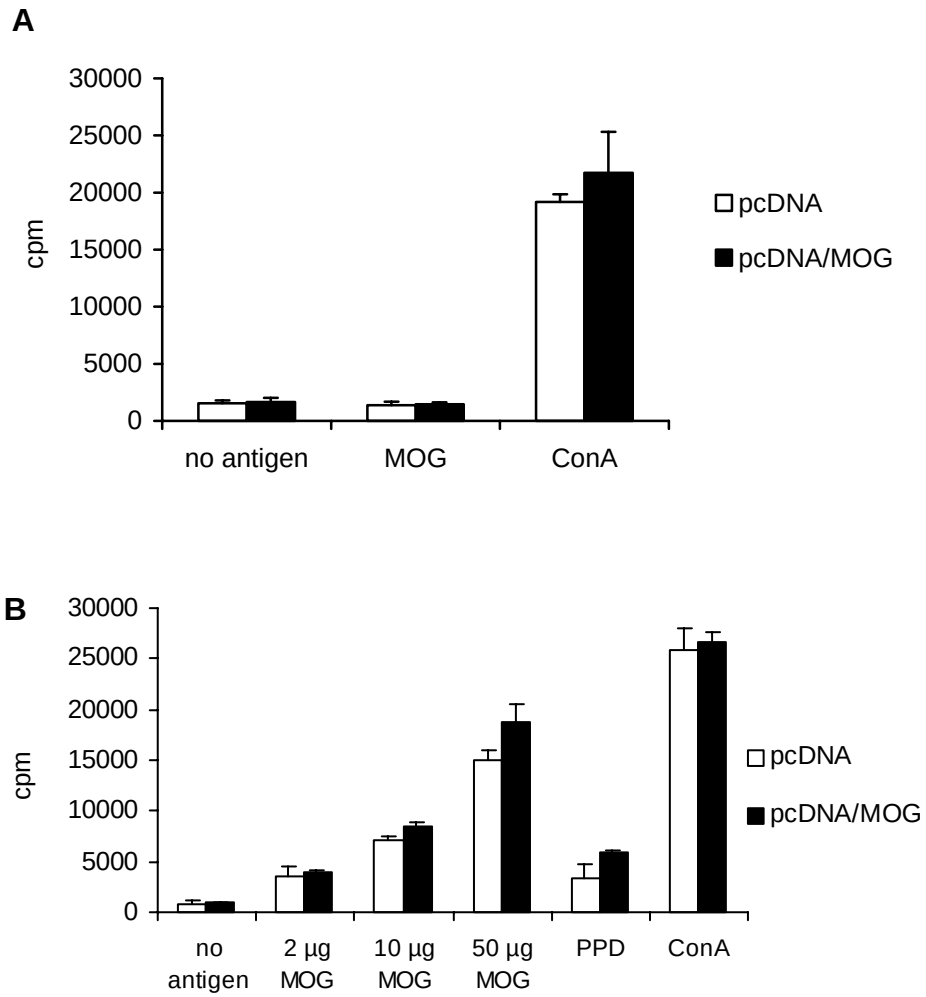

Figure 10: Specificity test of T cells following DNA vaccination
Specificity test of A) splenocytes isolated 5 weeks after vaccination with pcDNA or pcDNA/MOG and B) lymph node cells prepared 11 days after MOG^{Igd} immunisation of mice pretreated with pcDNA or pcDNA/MOG 28 days previously. T cell proliferation was determined by incorporation of ^{3}H-thymidine 72 h after incubation without antigen, with 2-50 µg/ml MOG^{Igd}, or with 10 µg/ml PPD or 2.5 µg/ml Con A. Assay was performed in triplicate.

Similarly, analysis of cytokine production by lymph node cells from mice immunised with MOG^{Igd} after MOG-DNA pretreatment provided no evidence for the induction of a "suppressive" Th2-like response. IFNγ and IL-10 production were measured by ELISA in the culture supernatant of lymph node cells stimulated by MOG^{Igd}. Although IFNγ production by cells from pcDNA/MOG and pcDNA pretreated mice did not differ significantly when cells were stimulated with 10 µg antigen, a significant increase ($p<0.01$) in IFNγ production by pcDNA/MOG pretreated cells was observed when they were stimulated with a higher antigen

concentration (50 µg MOG^{Igd}; Figure 11). There was no significant difference in IL-10 production at any antigen concentration (Figure 11). No IL-4 production was detected in either group of cells. These results indicate that treatment with MOG-DNA not only fails to suppress the MOG-specific T cell response but may even enhance a low grade Th1-like response in the treated animals.

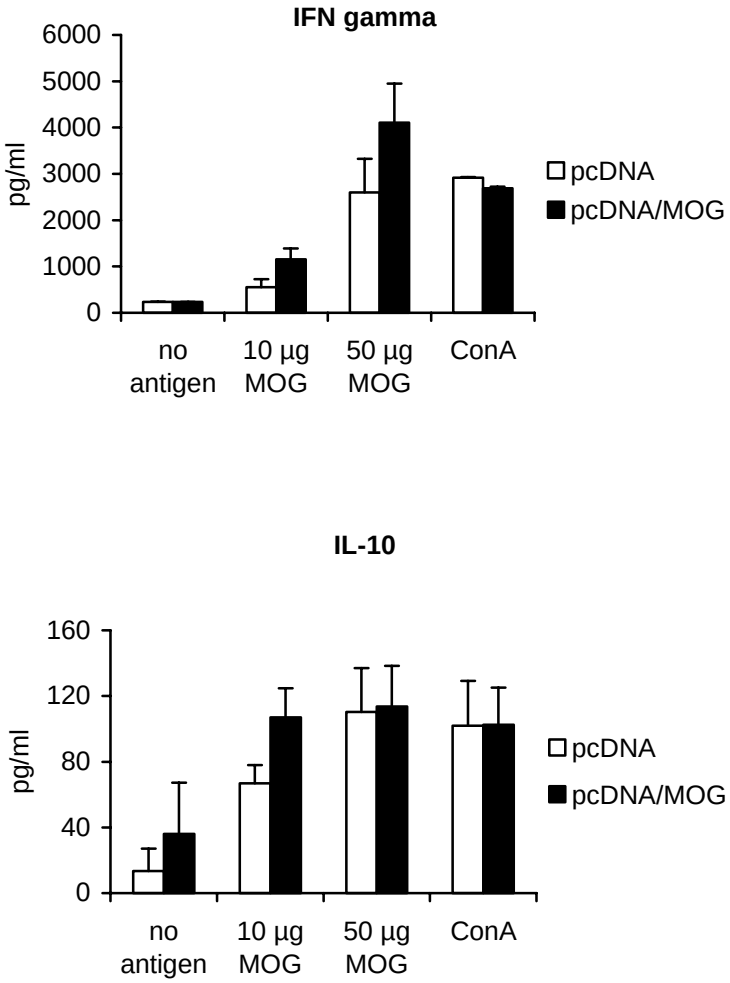

Figure 11: Influence of DNA vaccination on T cell cytokine production
Lymph node cells were prepared 11 days after MOG^{Igd} immunisation of mice pretreated with pcDNA or pcDNA/MOG 28 days previously. Secreted IFNγ and IL-10 in cell culture supernatant was measured by ELISA after 48 h in culture without antigen, with 10-50 µg/ml MOG^{Igd}, or with 2.5 µg/ml Con A. Assay was performed in triplicate and data are representative of 3 independent experiments.

6.5. Induction of EAE in DNA vaccinated SJL mice

6.5.1. Immunisation with MOG ^{Igd}

In the course of examining the T cell responsiveness of DNA vaccinated mice it was observed that pcDNA/MOG vaccinated animals appeared more susceptible to MOG-induced EAE than pcDNA vaccinated animals, suggesting that the vaccination protocol was potentially pathogenic. The clinical course of MOG-induced EAE was therefore studied in a larger number of animals pretreated with pcDNA/MOG or pcDNA. It was observed that in pcDNA/MOG vaccinated mice disease onset occurred two to three days earlier than in either nonvaccinated animals or mice vaccinated with pcDNA and followed a far more acute and severe clinical course. By day 15 p.i., 80 % of the animals pretreated with pcDNA/MOG had developed a clinical score of 5, as opposed to none of the controls (Figure 12). No disease was observed in pcDNA/MOG vaccinated mice immunised with CFA alone indicating that disease potentiation is dependent on an encephalitogenic challenge (Figure 12).

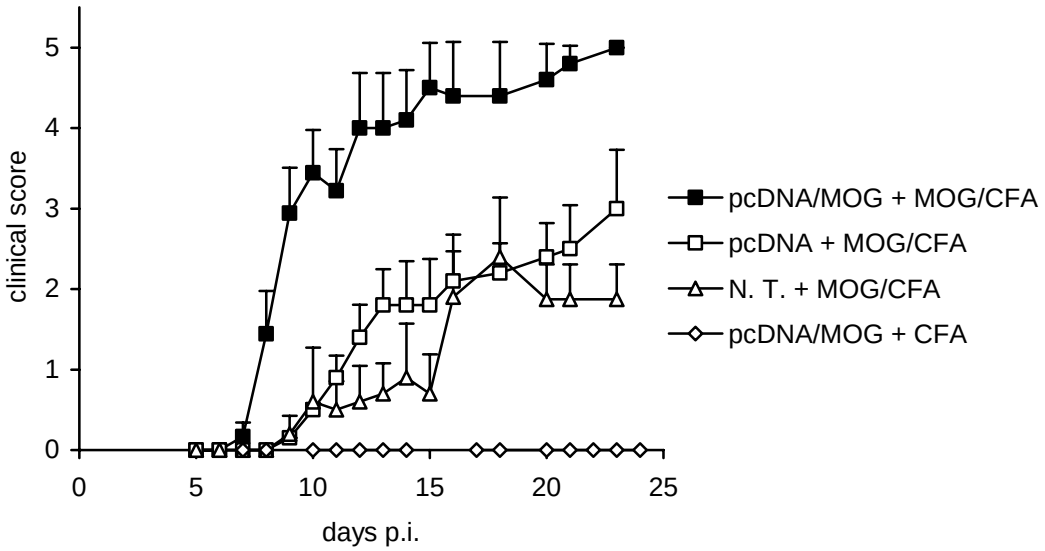

Figure 12: Clinical course of EAE induced by MOG^{Igd} following DNA vaccination
MOG-EAE was induced in SJL mice 4 weeks after pretreatment with pcDNA/MOG (■), pcDNA (□) or without pretreatment (N.T., Δ). Disease was induced by 200 µg MOG ^{Igd} in CFA. As a control, mice pretreated with pcDNA/MOG were immunised with CFA alone (◊). Data represent mean cumulative clinical score pooled from 2 independent experiments of 3 to 5 mice/group. Mice from one of the 2 experiments were sacrificed on day 10 for histopathological examination.

These clinical findings were paralleled by the histopathology of the CNS. Mice treated with control DNA developed only a low grade inflammatory response in the CNS by 10 days p.i. with little or no demyelination. In contrast even at this early time point extensive inflammation and demyelination was observed throughout the CNS of MOG-DNA vaccinated mice (Table 1 and Figure 13, histopathology by Dr. T. Berger).

Antigen	DNA	No of mice	Inflammation	Demyelination
MOG ^{Igd}	pcDNA	5	+	-
	pcDNA/MOG	5	+++	++
PLP 139-154	pcDNA	3	+	-
	pcDNA/MOG	6	+++	(+)
[1)] -	pcDNA	2	-	-
-	pcDNA/MOG	2	-	-
[2)] MOG $^{93-107}$	-	3	(+)	-

Table 1: Histopathology of EAE induced by MOG ^{Igd} or PLP $^{139-154}$ in DNA vaccinated animals
EAE was induced in SJL mice vaccinated with pcDNA or pcDNA/MOG by 200 µg MOG ^{Igd} or 100 µg PLP$^{139-154}$. Brain and spinal cord were examined for inflammation and demyelination 10-13 days after EAE induction.

Inflammation: (+): low grade meningitis, no perivascular inflammatory infiltrates. +++: severe meningitis, inflammatory infiltrates from medulla to forebrain.

[1)] 4 weeks after DNA vaccination alone, no signs of inflammation or demyelination were seen.
[2)] 11 days after immunisation with 100 µg MOG$^{93-107}$ (in the absence of DNA vaccination), a mild inflammation is present.

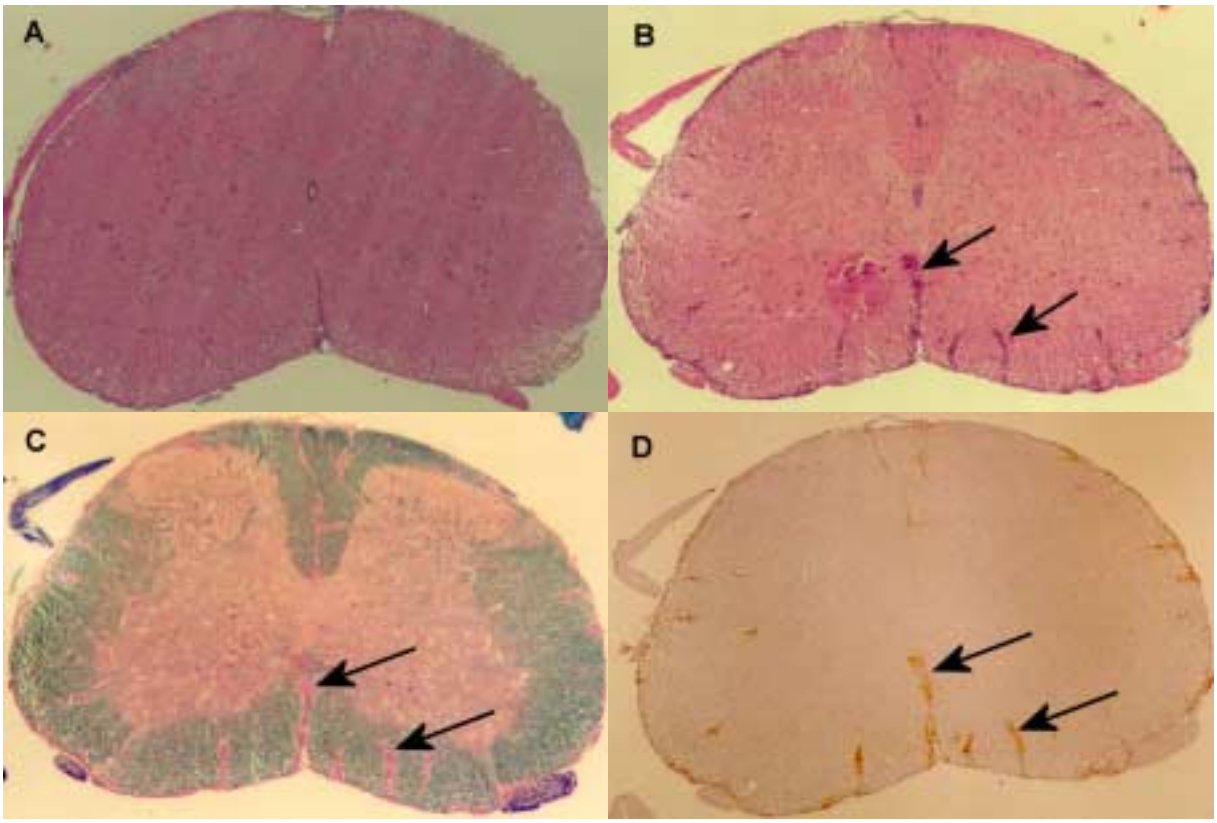

Figure 13: Histopathology of MOG-EAE in DNA vaccinated animals
EAE was induced in SJL mice with 200 µg MOG ^{1gd} 4 weeks after DNA vaccination with A) pcDNA or B-D) pcDNA/MOG. 10 µm spinal cord sections were prepared 10 days after EAE induction. Hematoxylin-eosin staining of A) a pcDNA pretreated mouse shows no inflammatory infiltrates and B) a pcDNA/MOG pretreated mouse shows perivascular infiltrates. C) The Luxol-Fast-Blue staining demonstrates destruction of myelin (stained blue) around the inflammatory infiltrates. D) An immunohistochemical staining for $CD4^+$ shows many $CD4^+$ T cells (stained brown) in the infiltrates.

6.5.2. Immunisation with MOG $^{93-107}$

Enhancement of disease activity by MOG-DNA vaccination was not restricted to MOG-EAE induced with the intact recombinant protein which induces both a demyelinating antibody response and a T cell response. Even stronger exacerbation was seen after immunisation with the weakly encephalitogenic MOG peptide, MOG$^{93-107}$ (Amor et al., 1994). In both control DNA pretreated and naive mice, a single immunisation with 100 µg MOG$^{93-107}$ induced a low grade inflammatory response in the CNS, but no obvious clinical signs of EAE (Table 1 and Figure 14A). In contrast, mice pre-treated with MOG-DNA developed severe clinical EAE following immunisation with MOG$^{93-107}$ (Figure 14A). Disease was first observed between days 7 and 9 p.i. and reached a peak of disease activity one to two days later at which time two of seven mice died. The mean maximum score was 3.9. The survivors failed to recover and exhibited a chronic neurological deficit for the remainder of the study period (Figure 14A).

As it has been reported that effects of DNA vaccination may differ according to time elapsed between vaccination and induction of EAE (Selmaj et al., 1999), the effect of DNA vaccination on EAE induced with MOG$^{93-107}$ several months after DNA vaccination was evaluated. Prior to induction of EAE, ELISA of the serum shows persistence of elevated anti-MOG antibody titers (Figure 8B). The clinical course of disease in pcDNA pretreated mice is relatively mild with an onset at day 12 and a slowly progressive clinical course to a mean maximum score of 2.5 (Figure 14 B). In contrast, in pcDNA/MOG pretreated mice, onset of disease is 3 days earlier with rapid progression to a score of 3-4. Interestingly, the clinical courses in both groups of animals are roughly parallel with a first peak of disease around day 14, followed by partial remission around day 20 and a more severe relapse that peaks after day 30 (Figure 14 B). These results demonstrate that MOG-DNA vaccination causes exacerbation of EAE even when disease is induced several months after DNA vaccination. The appearance of clinical signs in pcDNA pretreated mice contrasts with the data presented in Figure 14A in which pcDNA pretreated mice showed low grade inflammation of the CNS but no obvious clinical signs of disease. This is most likely to be attributed to the difference in age of the mice who in this experiment are 5 months older. It has been reported that older SJL mice are more susceptible to EAE (Kallen and Nilsson, 1989).

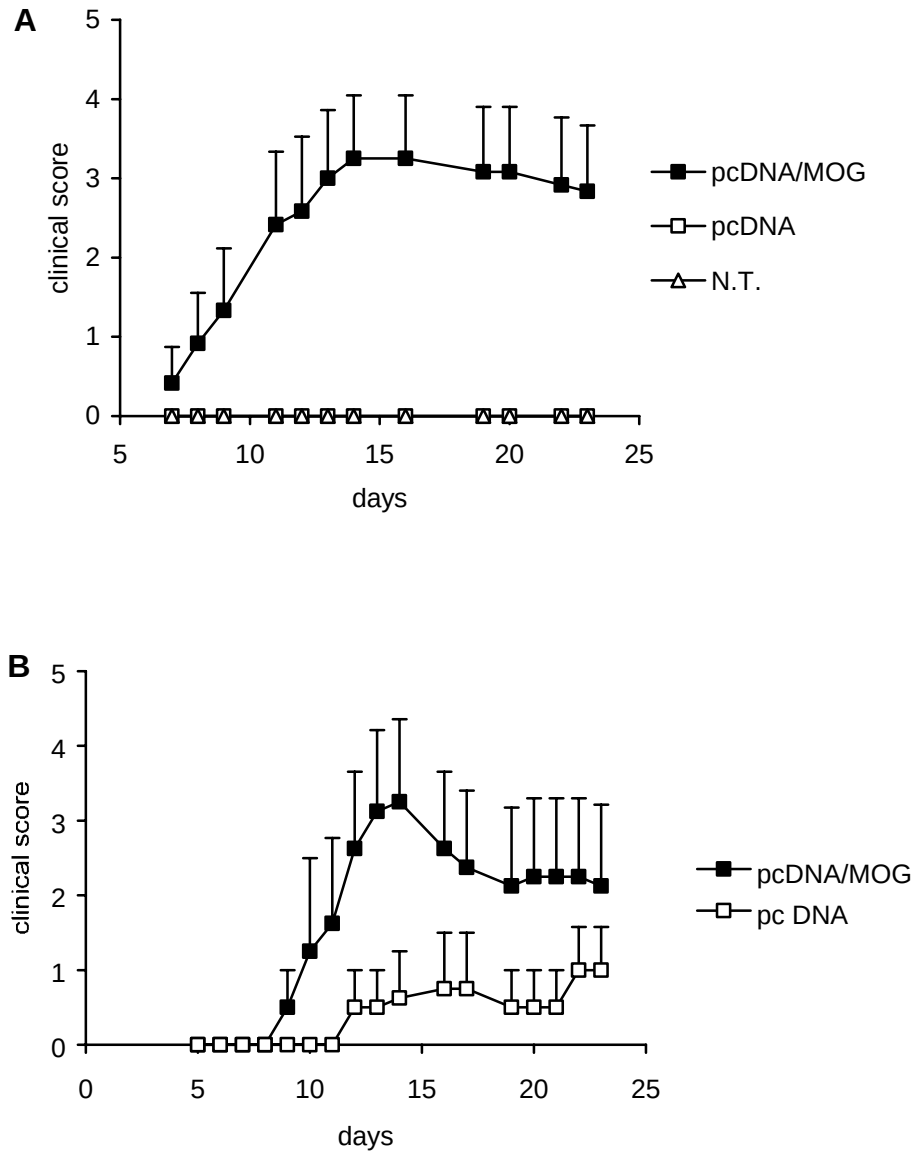

Figure 14: Clinical course of EAE induced by MOG $^{93-107}$
EAE was induced by 100 µg MOG $^{93-107}$ in SJL mice A) 4 weeks and B) 20-23 weeks after pretreatment with pcDNA/MOG, pcDNA or without pretreatment (N.T.). Data represent mean cumulative clinical score pooled from 4 mice/group. All pcDNA/MOG vaccinated mice had MOG-specific antibodies at the time of immunisation with MOG $^{93-107}$.

6.5.3. Immunisation with PLP$^{139-154}$

In order to investigate the antigen specificity of the effect of MOG-DNA vaccination on EAE, groups of control and pcDNA/MOG treated mice were immunised with an unrelated encephalitogen, the PLP peptide PLP$^{139-154}$. Strikingly, in PLP-induced EAE a similar enhancement of disease activity was observed as that seen in MOG-induced disease.

Untreated or control DNA immunised SJL mice developed a mild, chronic or relapsing remitting disease course following challenge with the PLP peptide $PLP^{139-154}$ (Figure 15 A). Histopathology at day 10 revealed an inflammatory response in the CNS but no demyelination (Table 1). In contrast, disease onset was 3 to 4 days earlier in MOG-DNA vaccinated mice and disease severity was increased (Figure 15 A). Analysis of the CNS showed extensive inflammation involving both brain and spinal cord and clear evidence of demyelination even at this early time point (Table 1). MOG-DNA vaccination therefore not only fails to suppress MOG-EAE, but actually enhances susceptibility to disease induced by an unrelated encephalitogen. The inability of control DNA to modify disease activity in any of these disease models eliminates the possibility that disease potentiation was due to immunostimulatory DNA sequences that non-specifically enhanced the immune response.

The exacerbation of both PLP- and MOG-induced EAE by MOG-DNA vaccination is reminiscent of the situation seen in transgenic mice containing an immunoglobulin heavy chain specific for MOG. These mice fail to develop EAE spontaneously but the circulating MOG-specific autoantibodies potentiate disease susceptibility and severity irrespective of the identity of the causative autoantigen (Litzenburger et al., 1998). Figure 15 B shows the typical clinical course of PLP peptide-induced EAE in knock-in and wild type mice. In MOG-Ig transgenic mice disease onset occurred around day 10 and rapidly progressed to a score of 3. It then continued in a relapsing remitting form for the rest of the observation period. In the wild-type littermates a very mild form of disease was observed with late onset after day 30. The parallels observed between MOG-DNA vaccinated mice and MOG-Ig transgenic mice suggested that exacerbation of EAE following DNA vaccination might be due to a pathogenic autoantibody response to immunisation with MOG-encoding DNA.

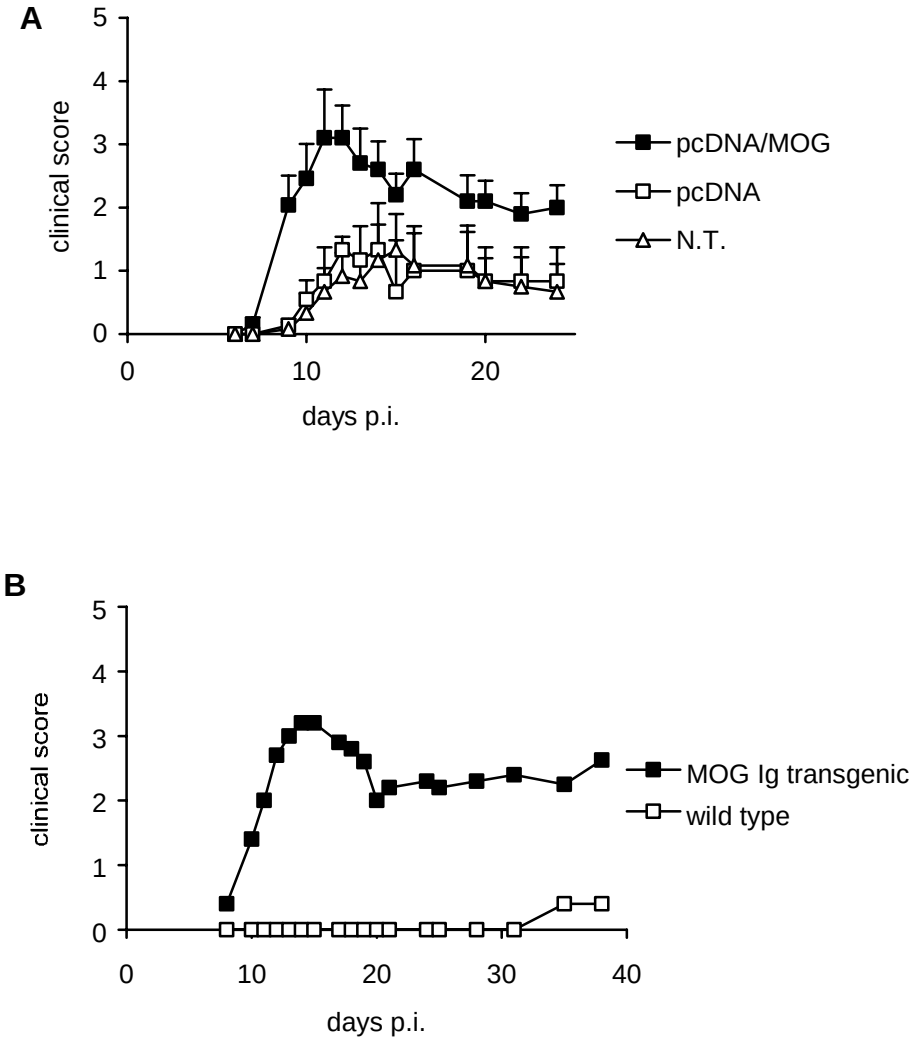

Figure 15: Clinical course of EAE induced by PLP$^{139-154}$ in MOG-DNA vaccinated mice and MOG Ig transgenic mice

A) EAE was induced in SJL mice 4 weeks after pretreatment with pcDNA/MOG, pcDNA or without pretreatment (N.T.) Disease was induced by 100 µg PLP $^{139-154}$. Data represent mean cumulative clinical score pooled from 2 independent experiments of 3 to 5 mice/group. Mice from one of the 2 experiments were sacrificed on day 10 for histopathological examination. B) EAE was induced with 200 µg PLP $^{139-154}$ in heterozygous MOG Ig transgenic mice and wild-type littermates. Mice were crossed back to the SJL background for 6-8 generations. Data represent mean cumulative clinical score of 5 mice/group.

6.6. Pathogenicity of the antibody response following DNA vaccination

6.6.1. Epitope specificity

To investigate the hypothesis that the exacerbation of EAE caused by MOG-DNA vaccination could be due to the autoantibody response induced, the MOG-specific antibody response was examined more closely with respect to its fine specificity and pathogenic potential.

The epitopes recognised by antibodies consist in some cases of a stretch of 6 to 20 aminoacids within a protein, independent from secondary or tertiary structure (linear epitopes). Other antibodies are directed against epitopes composed of aminoacids that are not directly adjacent in sequence and therefore depend on the conformation of the protein (conformation-dependent epitopes) (Colligan et al., 1991).

The epitope specificity of the antibody response induced by DNA vaccination was determined by ELISA against a panel of overlapping synthetic peptides (23-26 mer) spanning the complete extracellular domain of MOG. Interestingly, the MOG-specific antibodies did not recognise any of the peptides used (Figure 16A). In contrast, conventional immunisation with MOG^{Igd} in CFA induced an antibody response that recognised epitopes within the peptides 1 – 26 and 50 - 87, the latter being immunodominant (Figure 16B). These results suggest that the autoantibodies generated by MOG-DNA vaccination are directed against one or more conformational epitopes, as described previously for a panel of pathogenic demyelinating MOG-specific monoclonal antibodies (Brehm et al., 1999).

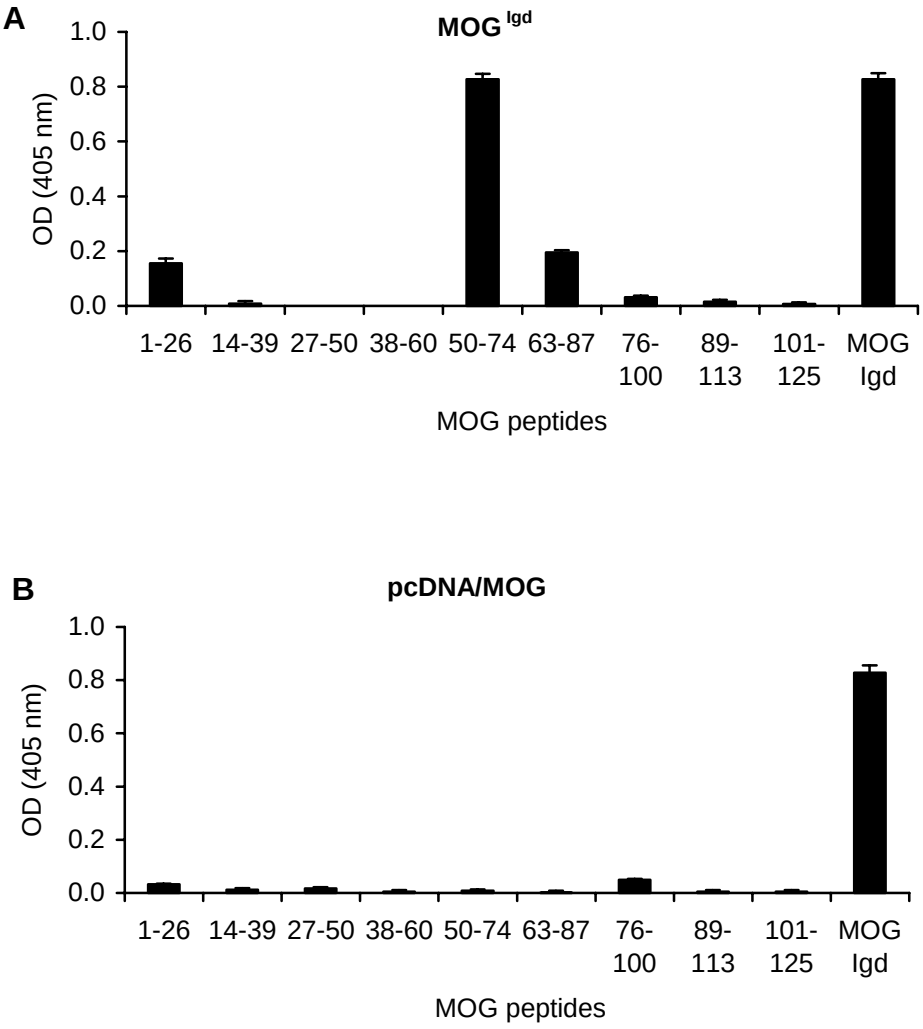

Figure 16: Epitope specificity of MOG-DNA vaccinated mice
Peptide specificity of serum antibodies from A) MOG^{lgd}-CFA immunised mice at day 21 and B) pcDNA/MOG vaccinated mice at day 28. Plates were coated with overlapping peptides (23 - 26-mer) spanning the extracellular domain of MOG. Sera were pooled from ≥ 3 mice at a dilution of 1/100 and assay was performed in triplicate. Anti-mouse IgG was used for detection. Background OD from naïve mouse serum was subtracted from test serum OD. Data are representative of at least 3 independent experiments.

6.6.2. Complement-dependent cytotoxicity

Binding to conformation-dependent epitopes of MOG^{lgd} exposed at the membrane surface is a prerequisite for MOG-specific autoantibodies to initiate demyelination *in vivo*, which is then mediated by a combination of complement and ADCC-dependent effector mechanisms (Piddlesden et al., 1991). The ability of the antibody response induced by MOG-

DNA vaccination to bind to and lyse Ag8/MOG, a transfected cell line expressing surface MOG (Figure 5), was therefore investigated. FACS analysis showed that anti-MOG autoantibodies induced by MOG-DNA vaccination bound specifically to the surface of the transfected cell line, but not untransfected myeloma cells (Figure 17). As expected from the absence of MOG-specific antibodies determined by ELISA, serum from control pcDNA vaccinated mice did not bind Ag8/MOG cells. The autoantibody response generated by MOG-DNA vaccination is therefore able to recognise the native extracellular domain of MOG exposed at the membrane surface, a pre-requisite if this response is to mediate the killing of MOG^+ targets *in vivo*.

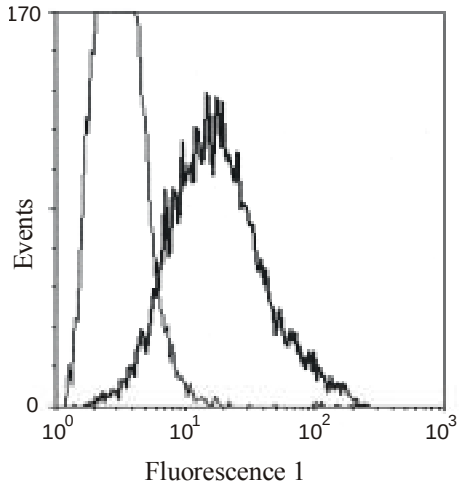

Figure 17: Specific binding of anti-MOG antibodies to Ag8/MOG transfectants
Ag8/MOG transfectants expressing surface MOG (solid line, see Figure 5) and Ag8 cells (dotted line) were incubated with serum from pcDNA/MOG vaccinated mice (day 28 p.v.) followed by Cy2-labeled anti-mouse IgG (5 µg/ml) and analysed by flow cytometry.
Fluorescence 1: detection of the Cy2-labeled second antibody.

The ability of this MOG-specific autoantibody response to induce a functional pathogenic mechanism was investigated *in vitro* by incubating Ag8/MOG cells together with serum from pcDNA/MOG vaccinated mice and rabbit complement. It was observed that the 1:100 dilution of serum was able to lyse approximately 80% of MOG-transfected myeloma cells in a dose and complement-dependent manner, but had no effect on untransfected myeloma target cells (Figure 18). In contrast, as anticipated from the absence of MOG-specific autoantibodies determined by both ELISA and FACS, equivalent dilutions of sera

from pcDNA immunised mice in the presence of rabbit complement had no influence on the viability of the MOG transfected targets (Figure 18).

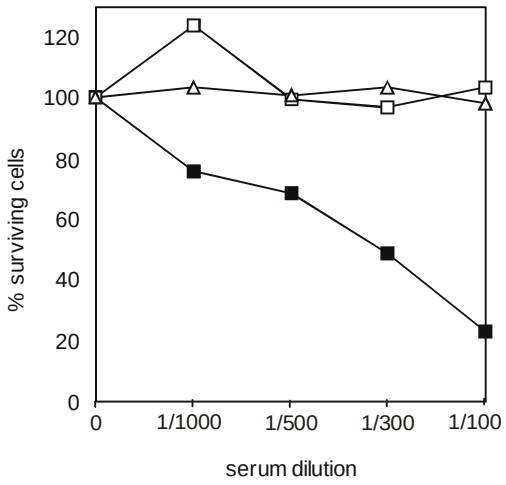

Figure 18: Complement-dependent lysis of MOG-positive cells induced by anti-MOG autoantibodies
Survival of MOG-transfected cells and untransfected control cells was measured after incubation with different dilutions of day 28 serum from pcDNA/MOG (■) or pcDNA (□) vaccinated mice in the presence of complement. 100 % survival is defined as cell survival with complement only, in the absence of test serum. Δ: untransfected Ag8 cells incubated with pcDNA/MOG serum.

6.7. DNA vaccination of mice transgenic for MOG-specific Ig

6.7.1. Clinical effect of DNA vaccination

The generation of a productive IgG2a antibody response following MOG DNA vaccination indicated the presence of a Th1-like T cell response although T cell proliferation and cytokine production were below detection levels. However, this T cell response alone was insufficient to induce clinical or histopathological signs of disease. It was hypothesized that the T cell response did not occur concomitantly with the DNA vaccination-induced B cell response and had already resolved at the time of high antibody production. To test whether the T cell response generated by DNA vaccination would be pathogenic in the presence of a high preexisting titer of MOG-specific antibodies, DNA vaccination was performed in transgenic knock-in mice containing an immunoglobulin heavy chain specific for MOG. These mice

have a large proportion of MOG-specific B cells and develop high titres of circulating anti-MOG antibodies (Litzenburger et al., 1998). The transgenic mice were generated in the 129sv/C57BL/6 strains and backcrossed to the SJL strain for 6 generations. No clinical signs of disease were noted for up to 6 weeks after MOG DNA vaccination in either transgenic or wild-type littermates. Animals were then immunised with MOG ^{Igd} 6 weeks after pretreatment with either pcDNA/MOG or pcDNA. No significant difference in incidence, onset or severity was noted between pcDNA/MOG and pcDNA pretreated animals: mice in both groups had relatively late onset of disease around day 17-20, before reaching a very severe mean maximum score of 4.1 to 4.2 (Table 2).

Pretreatment	Incidence	Day of onset	Mean maximum score
pcDNA/MOG	5/5	19.8 (+/- 5.2)	4.2 (+/- 1.0)
pcDNA	4/4	17.8 (+/- 5.9)	4.1 (+/- 0.8)

Table 2: MOG-EAE in MOG-Ig transgenic mice after pcDNA/MOG vaccination
Transgenic mice containing an immunoglobulin heavy chain specific for MOG were immunised with 200 µg of MOG ^{Igd} in CFA 6 weeks after vaccination with pcDNA/MOG or pcDNA. Mice were crossed back to the SJL background for 6 generations. Average (+/- standard deviation).

6.7.2. Effect of DNA vaccination on the B cell population

In view of the exacerbation of EAE seen in SJL mice after MOG-DNA vaccination, it was unlikely that DNA vaccination would induce tolerance to MOG in MOG-Ig transgenic mice. However, the effect of the expression of MOG outside the CNS by DNA vaccination on the large population of MOG-specific B cells in these mice was difficult to predict. B cells are known to function as antigen-presenting cells (Hayglass et al., 1986) and might contribute to induction of tolerance in MOG-Ig transgenic mice. Partial tolerance might be detected in the form of a temporary reduction of MOG-specific antibody titres or in a decrease of the proportion of MOG-specific B cells. To assess this possibility, the time course of serum antibody titers after DNA vaccination was determined in MOG-Ig transgenic mice. ELISA analysis showed a slight increase in MOG-specific Ig titers shortly after DNA vaccination that reached a plateau 2 weeks p.v. (Figure 19). This increase is however within the weekly

variability of MOG-antibody serum levels observed in these mice. Wild-type littermates show an increase in serum titers similar to that observed in SJL mice.

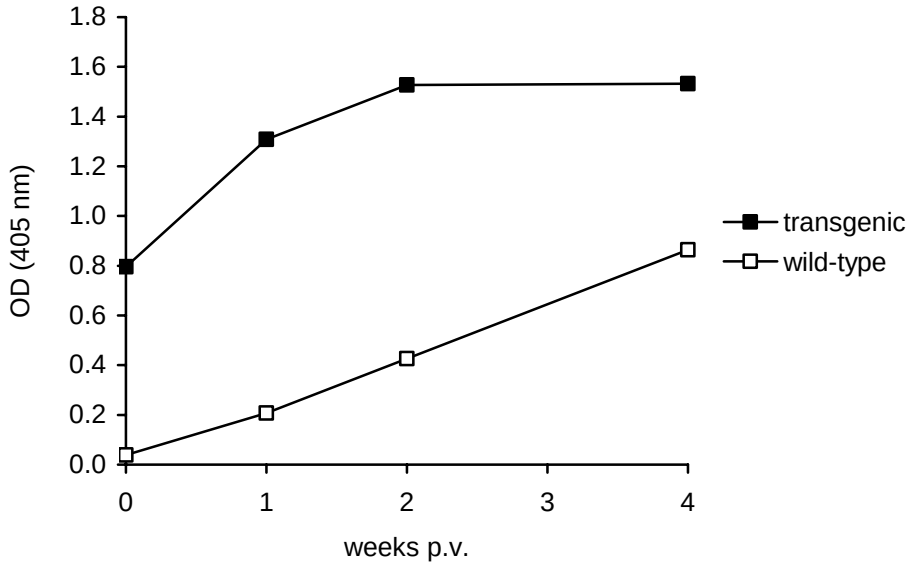

Figure 19: Anti-MOG antibody titers in MOG-Ig transgenic mice after DNA vaccination
ELISA determination of the time course of the MOG-specific Ig response following vaccination with DNA in MOG-Ig transgenic mice and wild-type littermates. Mice were crossed back to the SJL background for 6 generations. Sera were pooled from 4 to 5 mice vaccinated with pHSE/MOG and pipetted at a dilution of 1/100 on a MOG-coated ELISA plate. Anti-mouse total Ig was used for detection.

To analyse the effect of MOG-DNA vaccination on the B cell population, lymphocytes were isolated from blood of MOG-Ig transgenic mice at different time points after treatment with pcDNA/MOG or pcDNA. The proportion of transgenic B cells that specifically bind MOG (in naïve Ig-transgenics around 50 %) was assessed by FACS analysis. This experiment showed that both in pcDNA and pcDNA/MOG vaccinated mice the proportion of MOG-specific B cells remained between 45-60 % until 28 days after DNA vaccination (Figure 20). No signs of tolerance at the B cell level could therefore be detected in MOG-Ig transgenic mice.

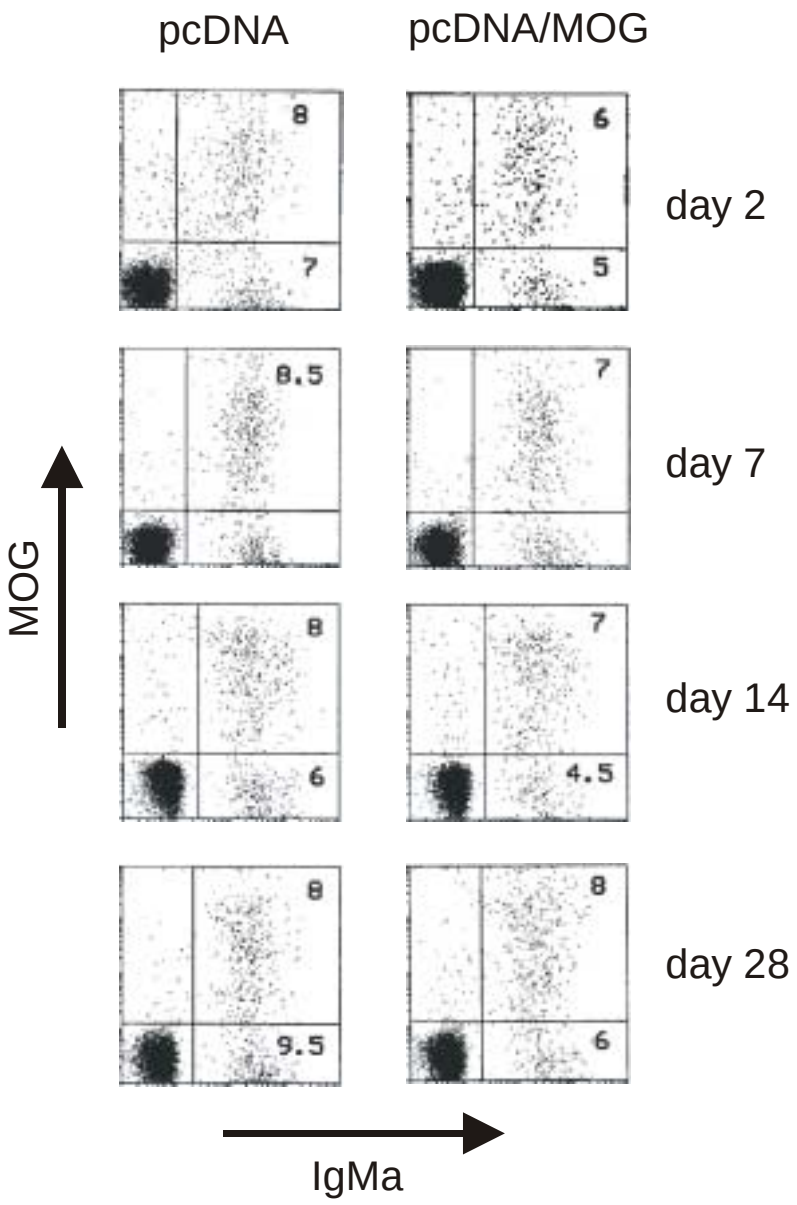

Figure 20: FACS analysis of MOG-specific B cells in MOG-Ig transgenic mice after DNA vaccination

Blood lymphocytes from pcDNA/MOG and pcDNA vaccinated heterozygous MOG-Ig transgenic mice were analysed by FACS 2, 7, 14 and 28 days after DNA vaccination. Cells were doubly stained with biotinylated MOG and anti-IgMa. IgMa detects the immunoglobulin "a" allotype, present only in the knock-in transgene but not in endogenous immunoglobulin. All transgenic IgM^+ B cells are in the right quadrants, with the MOG-specific B cells in the upper quadrant and non MOG-specific B cells in the lower quadrant. The proportion of MOG-specific B cells among all transgenic B cells remains between 45-60 % after DNA vaccination. Numbers represent the percentage of cells in the respective quadrant. Mice were crossed back to the SJL background for 6-8 generations. Data shown are representative of results obtained from ≥ 3 mice/group.

6.8. Effect of DNA vaccination in other mouse strains

6.8.1. MOG-specific antibody titers in different mouse strains

The most straightforward way to examine the role of MOG-specific autoantibodies generated by DNA vaccination in the exacerbation of EAE was to examine the effect of DNA vaccination on EAE induced in B cell deficient mice. In μmT mice, a targeted disruption of the membrane exon of the Ig μ chain gene arrests development of cells of the B lineage in the pre-B cell stage (Kitamura et al., 1991). Hence, these mice have no mature B cells and negligible circulating antibody levels. The strain was however available only on a C57BL/6 background. In preliminary experiments it became obvious that wild-type C57BL/6 mice produced only very low levels of MOG-specific antibodies after DNA vaccination and that these μmT mice could therefore not be used to analyse the role of MOG-specific autoantibodies in the exacerbation of EAE.

It was however intriguing that the C57BL/6 strain which is susceptible to MOG-EAE did not produce an antibody response to MOG following DNA vaccination. This strain is generally known to be a good responder to DNA vaccination with DNA encoding bacterial and viral proteins. To investigate this further, DNA vaccination was performed simultaneously in C57BL/6 and SJL mice that are susceptible to MOG-induced EAE, but also in the mouse strains 129sv and Balb/c that are resistant to EAE. 4 weeks after DNA vaccination, mice were tested individually for circulating MOG-specific IgG. Balb/c mice had high levels of anti-MOG antibodies comparable to those obtained in SJL mice. 129sv mice had low levels of anti-MOG antibodies compared to those of SJL mice. Interestingly, none of the C57BL/6 mice tested had an antibody response above background levels (Figure 21A).

The antibody isotypes were also assayed to see if other strains than SJL showed an antibody response dominated by the IgG2a isotype indicative of a Th1-like response. The ratio of IgG1 to IgG2a was identical in Balb/c and SJL mice (0.3) (Figure 21B), indicating a marked bias toward IgG2a in these strains. 129sv mice showed an even more skewed response toward IgG2a, as no IgG1 was detected. The ratio of IgG1 to IgG2a in SJL mice immunised with MOG ^{Igd}-CFA was 0.9, indicating similar amounts of both isotypes and no marked bias.

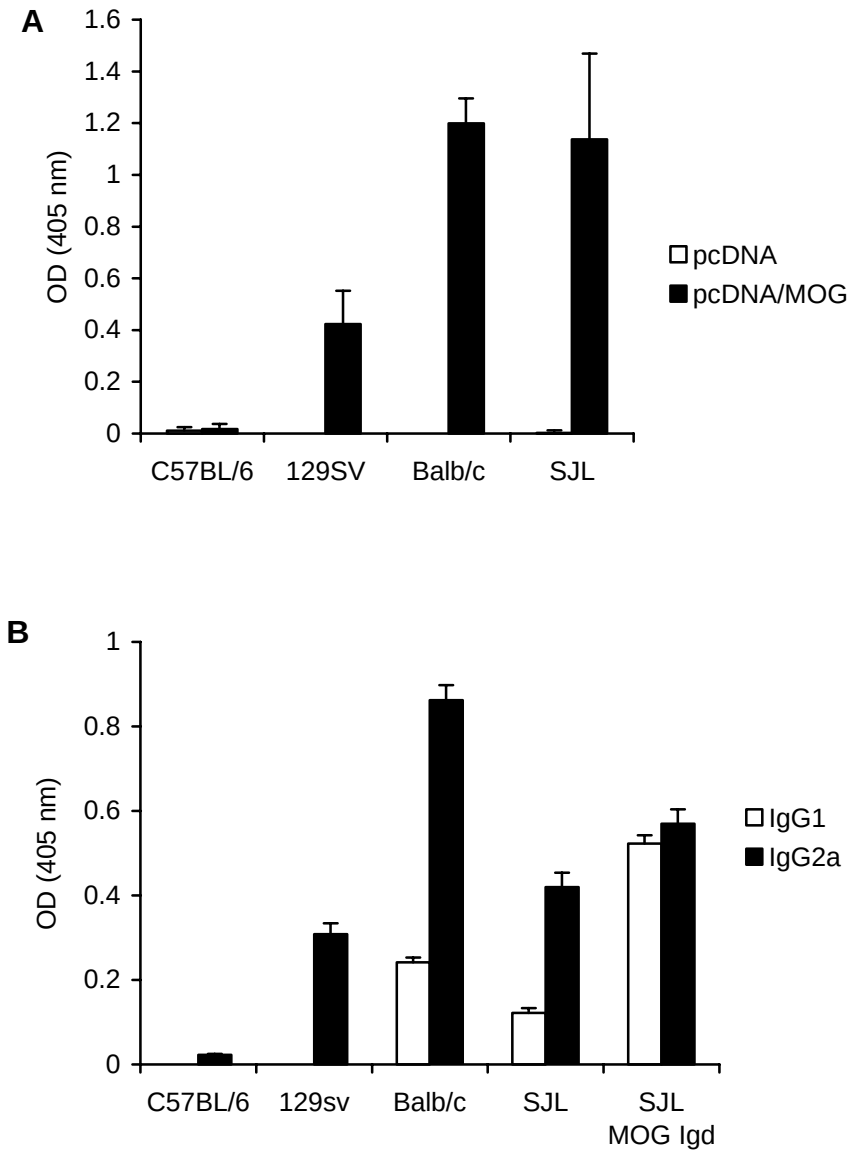

Figure 21: MOG-specific antibody titers in different mouse strains
A) MOG-specific IgG determined by ELISA in serum of pcDNA and pcDNA/MOG vaccinated mice of different strains 28 days p.v. Data represents the average of 5 mice/group assayed individually (duplicates). B) MOG-specific IgG1 and IgG2a in serum of pcDNA/MOG vaccinated mice of different strains 28 d.p.v. and in SJL mice immunised with MOG ^{Igd}/CFA 14 d.p.i.

The ability of C57BL/6 mice to mount a normal antibody response to MOG was then shown by immunising C57BL/6 mice with MOG ^{Igd} in IFA or CFA. Serum IgG titers were determined 28 days after immunisation. At this time MOG-specific antibody titers were similar in C57BL/6 and SJL mice immunised with MOG ^{Igd} in CFA (Figure 22).

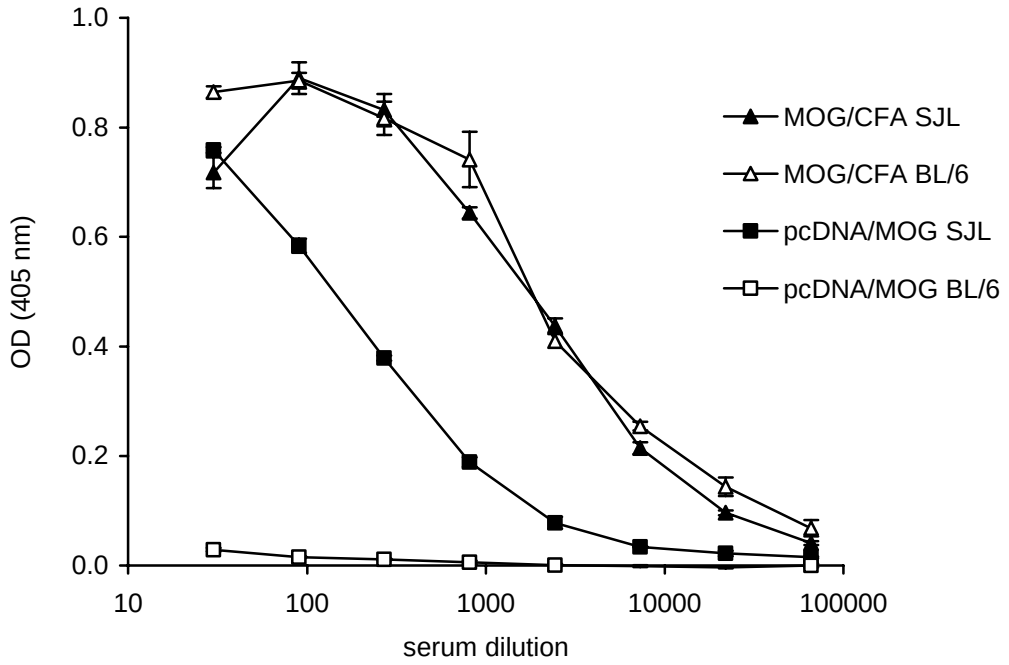

Figure 22: Induction of MOG-specific antibodies in C57BL/6 mice by MOG ^{Igd} immunisation
MOG-specific antibody titers in SJL and C57BL/6 mice 28 days after vaccination with pcDNA/MOG or immunisation with MOG^{Igd} in IFA or CFA. Serial dilutions of sera pooled from ≥ 3 mice were pipetted onto a MOG-coated ELISA plate. Anti-mouse IgG was used for detection. Assay was performed in triplicate.

6.8.2. MOG-EAE in DNA vaccinated C57BL/6 mice

To see if pcDNA/MOG vaccination had any effect on the clinical course of EAE in C57BL/6 mice, disease was induced in C57BL/6 mice 4 weeks after DNA vaccination with pcDNA, pcDNA/MOG or untreated mice. Disease onset was on days 11-12 in all groups and progressed rapidly to peak of disease severity around day 18 (Figure 23). The course was then relapsing-remitting. Disease course was similar in all groups of animals and no exacerbation was seen in pcDNA/MOG pretreated mice.

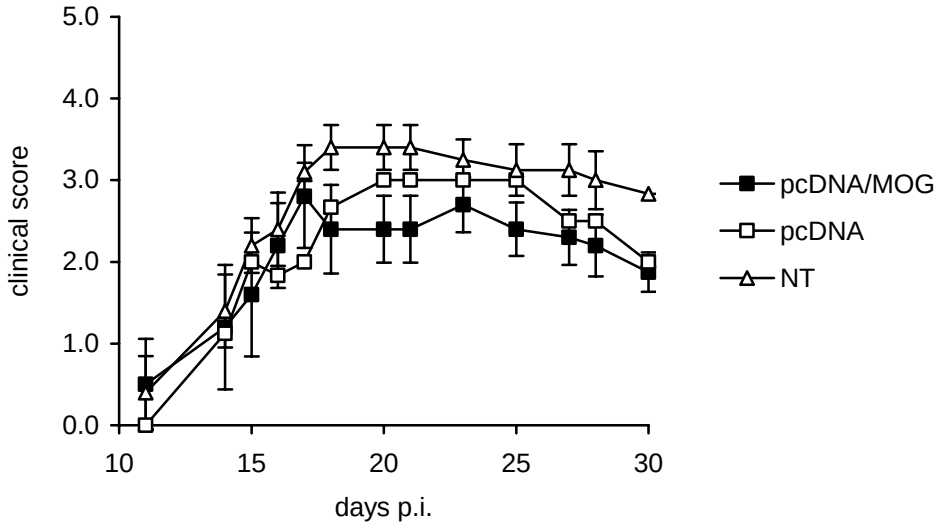

Figure 23: Clinical course of EAE in C57BL/6 mice after DNA vaccination
EAE was induced 4 weeks after pretreatment with pcDNA/MOG, pcDNA or without pretreatment (N.T.) Disease was induced by immunisation with 200 µg MOG $^{35-55}$, the dominant encephalitogenic peptide for C57BL/6 mice. Data represent mean cumulative clinical score of 5 mice/group.

7. DISCUSSION

7.1. Advantages of MOG-DNA vaccination

DNA vaccination, the in vivo administration of naked DNA molecules encoding the antigen of interest, presents many advantages over conventional immunisation techniques. DNA vaccination results in expression of antigen by host cells, inducing production of protein with native posttranslational modifications and conformation that can elicit antibodies of optimal specificity (Attanasio et al., 1997). In vivo expression of antigen efficiently generates a long-lasting immune response including $CD8^+$ cytotoxic T cells, $CD4^+$ T helper cells and antibody-secreting B cells (Donnelly et al., 1997). Adjuvant effect is provided by immunostimulatory CpG sequences within the plasmid itself (Roman et al., 1997; Chu et al., 1999). In addition, DNA vaccines are readily produced at high purity and facilitate the combination of several immunogens into one administered preparation. One major safety concern is the possibility that the DNA may integrate into the host genome. As the plasmids used for vaccination do not include an origin of replication that is functional in eucaryotic cells, they cannot replicate in the mammalian host, thus reducing the risk of integration. Furthermore, the transfected cells (mainly myocytes and dendritic cells) are for the most part non-dividing and the constructs injected have very low sequence homology with host DNA, making integration by homologous recombination highly unlikely. In practice, integration of plasmid DNA into the host genome is rarely observed and is estimated to occur at a frequency that is 3 orders of magnitude less than that of spontaneous mutation (Wolff et al., 1990; Donnelly et al., 1997). So far, DNA vaccination has proved very safe in both experimental and clinical studies.

The efficacy of vaccination with DNA encoding self-antigens in suppressing EAE was first demonstrated by vaccination with DNA encoding peptides derived from either of the major target antigens, MBP and PLP (Ramshaw et al., 1997; Lobell et al., 1998; Lobell et al., 1998; Ruiz et al., 1999). EAE induced by immunisation with either of these autoantigens is frequently used as an animal model of multiple sclerosis, but unlike the human disease is purely T cell mediated (Martin et al., 1992). In contrast the immunopathogenesis of multiple sclerosis is far more complex and involves both T and B cell responses to myelin autoantigens (Lassmann et al., 1998; Genain et al., 1999a). This study investigated the effect of DNA

vaccination on MOG-induced EAE, an animal model which also involves antibody-dependent immune effector mechanisms and more closely reproduces the immunological and pathological complexity of MS (Genain et al., 1999a; Storch et al., 1998b). Furthermore, MOG is an important target for autoimmune T and B cell responses in multiple sclerosis (Sun, 1993; Kerlero de Rosbo et al., 1993; Reindl et al., 1999; Lindert et al., 1999; Ben-Nun et al., 1996; Genain et al., 1999a).

7.2. Expression of MOG outside the CNS by DNA vaccination

MOG is a CNS-specific myelin membrane glycoprotein synthesised by oligodendrocytes that is preferentially integrated into the outermost surface of the myelin membrane (Linington et al., 1984; Brunner et al., 1989; Gardinier et al., 1992). It has not been detected in either the peripheral nervous system or in lymphoid organs (Gardinier et al., 1992; Litzenburger, 1997). Analysis by RT-PCR in this work showed that, additionally, MOG is not normally expressed in striated muscle (Figure 6). As a CNS-specific protein, MOG is therefore shielded from contact with cells of the immune system by an anatomical barrier, the blood-brain-barrier, implying that tolerance to MOG is most probably achieved not by anergy or deletion, but by clonal ignorance. Indeed MOG-specific lymphocytes are a normal component of the T cell repertoire of healthy individuals (Kerlero de Rosbo et al., 1993; Kerlero de Rosbo et al., 1997), and transgenic mice bearing a large proportion of MOG-specific B cells remain healthy, suggesting that tolerance is maintained by clonal ignorance in these mice (Litzenburger et al., 1998). However, if activated by an encephalitogenic challenge these autoreactive T cells can cross the blood-brain-barrier and induce a full-blown inflammatory response in the CNS (Martin et al., 1992; Litzenburger et al., 1998).

Although the most efficient way to ensure self-tolerance is by deletion of developing self-reactive lymphocytes in the central lymphoid organs (thymus and bone marrow), tolerance can also be induced upon encounter of autoreactive lymphocytes with self-antigen in the periphery. As a rule, naive T cells that recognise self-antigens presented by tissue cells in the periphery do not receive the necessary costimulatory signals and are either functionally inactivated (Schwartz, 1996) or deleted by programmed cell death (Miller and Basten, 1996), thereby ensuring tolerance to tissue-specific autoantigens not present in the central lymphoid organs. In a similar way, mature B cells that recognise self-antigens in the periphery are deleted or rendered anergic if they do not simultaneously receive the appropriate

costimulatory signal from helper T cells (Nossal and Pike, 1980; Goodnow et al., 1988). In this study, DNA vaccination was used to express MOG in host cells outside the CNS. The aim was to examine the effect of ectopic MOG production on the MOG-specific immune response and to investigate a possible tolerance induction by the presence of antigen on tissue cells in the periphery.

The DNA construct used for vaccination contains the full-length mouse MOG cDNA including the signal sequence, allowing integration of the mature protein into the plasma membrane. This could be clearly demonstrated by FACS analysis of a mouse myeloma cell line transfected with the same DNA vector used for vaccination (Figure 5). Following intramuscular injection of MOG-DNA, it was however not possible to detect MOG-expressing cells in muscle either by Western blotting or by immunohistochemistry. Expression of the encoded antigen below the threshold of detection is not uncommon following DNA vaccination (see introduction). Vaccination studies performed with reporter constructs encoding chloramphenicol acetyl transferase, β-galactosidase and luciferase suggest a maximum transfection efficiency of 1-5 % of muscle fibers in the immediate vicinity of the injection site and the synthesis of 100-500 pg protein /muscle injected (Wolff et al., 1990; Ulmer et al., 1993; Pardoll and Beckerleg, 1995). MOG mRNA transcripts and plasmid DNA were however detected by RT-PCR in muscle for up to 14 days following MOG DNA vaccination (Figure 6, Figure 7). In addition to local expression in the muscle, migration of transfected cells to lymphoid organs has also been described (Casares et al., 1997; Torres et al., 1997), but in this study no mRNA transcripts or plasmid DNA were detected in lymphoid tissue at any time point investigated. Furthermore, no histological signs of inflammation were detected in injected muscle for up to 14 days after MOG-DNA vaccination although immune-mediated destruction of transfected muscle fibres following DNA vaccination has been described by others (Davis et al., 1997).

The induction of a long-lasting MOG-specific antibody response provided indirect evidence that MOG was expressed in vivo following DNA vaccination. Circulating MOG-specific antibodies were first observed two weeks after vaccination and titres reached a maximum two weeks later, then slowly declined. Antibodies were still detectable for at least 5 months after vaccination (Figure 8). Since the half-life of mouse circulating antibodies is approximately 3 weeks (Pollock et al., 1990), the slow decrease of MOG-specific antibody

titres suggests that the antigen persists in vivo for several months after vaccination. However, as no MOG transcripts were detected by RT-PCR later than 14 days after vaccination, it is probable that this reflects the persistence of antigen-antibody complexes on follicular dendritic cells in the lymph nodes rather than de novo synthesis.

In DNA vaccination, the specific character of the developing immune response can be influenced by several factors including route of immunisation and cellular location of antigen (Donnelly et al., 1997; Feltquate et al., 1997). While intramuscular injection induces mainly antibodies of the IgG2a isotype and production of IFNγ indicative of a Th1-like response, use of the gene gun shifts the antibody profile towards IgG1 and increases IL-4 production, suggesting skewing toward a Th2-like T cell response (Pertmer et al., 1996; Feltquate et al., 1997). Interestingly, skewing of the immune response according to the route of DNA vaccination even appears to overrule a general disposition to mount a Th1 or Th2 response. Comparison of DNA vaccination in Balb/c and C57BL/6 mice that are genetically predisposed to develop a Th2 or Th1 immune response, respectively, demonstrated that DNA vaccination can overcome the inherent biases in the immune response of these two mouse strains (Feltquate et al., 1997). The cellular location of the encoded antigen also influences the type of immune response, as expression of a membrane-bound antigen by DNA vaccination induces a Th1 like response, while vaccination with DNA encoding the same protein in secreted form can result in either a Th2 or a mixed Th1/Th2 response (Cardoso et al., 1996; Haddad et al., 1998; Lewis et al., 1999). In the present study, use of a construct encoding a transmembrane protein and vaccination by the intramuscular route would predict a response of the Th1 type. This was indeed the case as the antibody response induced by MOG DNA vaccination in SJL mice was predominantly of the IgG2a isotype, suggesting a Th1 like response (Figure 9).

7.3. Effect of MOG-DNA vaccination on the T cell response

The production of a specific antibody response following DNA vaccination implies the induction of helper T cells. Indeed, a general feature of DNA vaccination is the generation of T cell help by priming of $CD4^+$ T cells (Donnelly et al., 1997; Tighe et al., 1998). This can be demonstrated by the specific proliferation of lymph node cells in response to an MHC II restricted peptide following vaccination with DNA encoding this epitope (Zhong et al., 1996). The helper T cell response induced by intramuscular DNA vaccination is generally Th1-like, a

fact which may be due to the particular adjuvant properties of bacterial DNA (see introduction). This is indicated by the predominant IgG2a antibody response usually observed following DNA vaccination (Manickan et al., 1995; Cardoso et al., 1996; Aberle et al., 1999), and by the in vitro secretion of Th1-associated cytokines by splenocytes and lymph node cells in response to antigen stimulation (Manickan et al., 1995; Inchauspe et al., 1997; Ulmer et al., 1998). T cell lines generated from peripheral blood lymphocytes of rhesus monkeys following vaccination with DNA encoding the HIV-1 env protein all displayed $CD4^+$ markers (Lekutis et al., 1997) and all cell lines obtained secreted IFNγ and TNFα without appreciable IL-4 production, again implying a Th1 like response. $CD4^+$ T cells also play a role in induction of the CTL response seen after vaccination with DNA encoding viral antigens (Ulmer et al., 1993). Following intramuscular vaccination with DNA encoding ovalbumin peptides restricted for either MHC class I or MHC class II molecules, the CTL response was present only if vaccination was performed with both MHC I and MHC II epitopes, but not with an MHC I epitope alone, indicating that the CTL response following DNA vaccination is highly dependent upon the generation of $CD4^+$ T cell help (Maecker et al., 1998). In addition, the Th1 mediated protection against viral infection conferred by DNA vaccination can be transferred by $CD4^+$ T cells (Manickan et al., 1995; Ulmer et al., 1998).

In this context, the effect of vaccination with DNA encoding myelin autoantigens on EAE, a Th1 T cell mediated autoimmune disease, is not yet clearly defined. In some studies, suppression of EAE associated with partial suppression of the Th1 T cell response was observed following DNA vaccination. Vaccination with DNA encoding MBP or PLP peptides before the encephalitogenic challenge reduced the subsequent IgG2a response and suppressed antigen-specific secretion of Th1 cytokines by lymph node cells (Lobell et al., 1998; Ruiz et al., 1999). However, transfer of lymph node cells from vaccinated animals to naive animals did not transfer disease suppression (Ramshaw et al., 1997). In contrast, in a conflicting report a similar vaccination procedure using the same PLP peptides actually increased the specific T cell response after PLP peptide challenge and exacerbated rather than suppressed EAE (Tsunoda et al., 1998). In the present work, vaccination with MOG-DNA did not suppress the MOG-induced T cell proliferation or cytokine secretion induced by challenge with MOG^{Igd} (Figure 10, Figure 11). Indeed, a slight increase in both proliferation and IFNγ secretion could be observed in some experiments. Furthermore, the presence of an IgG2a antibody response following MOG-DNA vaccination suggested induction rather than suppression of a MOG-

specific Th1 response. Analysis of the T cell response 5 weeks after MOG-DNA vaccination showed no MOG-specific proliferation or cytokine secretion by splenocytes (Figure 10), indicating that the frequency of MOG-specific Th1 cells was either very low or that this population was only transiently present, a finding that correlates with the fact that MOG-DNA vaccination alone did not induce any clinical or histopathological signs of EAE (Table 1). Indeed, disease was not elicited by MOG-DNA vaccination even in the presence of preexisting pathogenic anti-MOG antibody responses in anti-MOG Ig transgenic mice (Litzenburger et al., 1998); Figure 15B). This suggests that the number of MOG-specific effector Th1 T cells was too low to induce EAE, implying that a minimum number is necessary at a given time for disease induction. Alternatively, MOG-DNA vaccination may stimulate a different Th1 T cell population than that involved in disease induction. It is also possible that in Ig transgenic mice, cells that express MOG in the periphery are recognised by the preexisting anti-MOG antibodies and rapidly eliminated, thereby preventing the induction of a significant MOG-specific T cell response.

The inability of DNA vaccination to suppress the MOG-specific T cell response is difficult to explain in the context of reports that clearly demonstrate suppression of T cell responses to two other myelin antigens. It is possible that this may reflect differences related to the nature of the constructs used, the sites and route of vaccination and timing of the challenge with encephalitogen, rather than a MOG-specific effect *per se*. Thus in contrast to the present study, the suppressive effects of PLP-peptide encoding DNA on EAE was investigated after 2 injections of DNA followed by challenge with PLP peptide only 10 days later (Ruiz et al., 1999). Additional methodological differences include the induction of muscle regeneration by bupivacain 2 days before the first DNA injection and measurement of T cell responses only after recovery from the acute phase of disease. Such variations in the vaccination protocols make it difficult to make any generalised interpretation as to how DNA vaccination influences autoantigen-specific T cell responses.

It may be speculated that fundamental differences in the tissue-specific expression of MBP and PLP as opposed to MOG may play some role in determining the outcome of DNA vaccination in these EAE paradigms. Isoforms of MBP and PLP are not only expressed in the nervous system but are also present in the thymus and secondary lymphoid tissue (Fritz and Kalvakolanu, 1995). The expression of these proteins outside the nervous system has a major

influence on the MBP- and PLP-reactive T cell repertoires. Indeed, a recent study demonstrated that the naturally occurring expression of PLP in the thymic epithelium shapes the PLP autoreactive T cell repertoire and maintains tolerance to PLP in C57/B6 mice (Klein et al., 2000). In contrast the CNS-specific expression of MOG implies that the MOG-reactive T cell repertoire is not exposed to MOG in the periphery and tolerance is maintained solely by clonal ignorance. Vaccination with MOG-DNA can therefore be compared to vaccination with DNA constructs encoding "foreign" antigens such as viral proteins. In both paradigms the antigen is seen as foreign and vaccination induces a Th1 like response associated with a strong B cell component (Donnelly et al., 1997; Tighe et al., 1998).

As studies on EAE and MS have generally focused on helper T cell and B cell responses, little is known about the role of autoreactive $CD8^+$ T cells in these diseases. Previous reports suggest that $CD8^+$ T lymphocytes may participate as both effectors and regulators in EAE. Thus, $CD8^+$ knock-out mice have milder MBP-induced EAE but more frequent relapses than wild-type mice and similar results were obtained in $CD8^+$-depleted mice (Koh et al., 1992; Jiang et al., 1992). The effector function of $CD8^+$ T cells may predominate in the early phase of EAE where these cells are found in inflammatory infiltrates in the CNS (Koh et al., 1992; Kalman et al., 1995). In contrast, $CD8^+$ T cells may downregulate inflammation in the chronic/relapsing phase of disease either through cytokine secretion or by directly killing encephalitogenic $CD4^+$ T cells (Sun et al., 1988; Koh et al., 1992; Jiang et al., 1992). As DNA vaccines encoding pathogens are efficient inducers of $CD8^+$ responses (Donnelly et al., 1997; Tighe et al., 1998), it is interesting to consider a possible role for $CD8^+$ T cells following vaccination with DNA encoding self-antigens. DNA encoding an MBP peptide suppresses MBP-induced EAE even in $CD8^+$-depleted rats, indicating that protection is not mediated by $CD8^+$ lymphocytes (Lobell et al., 1998). The possible exacerbating role of autoantigen-specific $CD8^+$ T cells has however not been investigated. In this context, it would be interesting to study the induction and functional significance of MOG-specific $CD8^+$ T cells following MOG-DNA vaccination. These cells might influence the course of disease through pro-inflammatory cytokine secretion or through direct cytotoxicity.

7.4. Mechanism of exacerbation of EAE following MOG-DNA vaccination

Rather than enhancing tolerance and decreasing susceptibility to EAE, MOG-DNA vaccination not only exacerbated disease induced with both MOG^{Igd} and the encephalitogenic peptide MOG^{93-107}, but also exacerbated EAE induced by an unrelated encephalitogen, the peptide $PLP^{139-154}$. This is in striking contrast to results obtained using DNA constructs encoding MBP or PLP peptides that suppress T cell responsiveness to the target autoantigen and have a marked beneficial effect on disease activity (Lobell et al., 1998; Ruiz et al., 1999). One study reported exacerbation of EAE induced 4 weeks after vaccination with PLP-DNA, but amelioration of EAE induced at a later time point (10 weeks p.v.) (Selmaj et al., 1999; Selmaj et al., 1999). In the present work no such difference was observed, as exacerbation of MOG-induced EAE was seen after encephalitogenic challenge at both 4 and 19-23 weeks post vaccination (Figure 15). On histopathological examination, mice vaccinated with MOG-DNA before induction of MOG-EAE had widespread inflammatory infiltrates accompanied by extensive demyelination (Table 1 and Figure 13).

The most probable mechanism of exacerbation of disease following MOG-DNA vaccination is through the action of the Th1 associated MOG-specific antibody response. The demyelinating activity of MOG-specific autoantibodies is well established in EAE and a similar response is now implicated in the immunopathogenesis of demyelination in MS (Schluesener et al., 1987; Linington et al., 1988; Litzenburger et al., 1998; Storch et al., 1998a; Genain et al., 1999a). The pathogenicity of MOG-specific antibodies is attributable to the particular ultrastructural localisation of the protein. The compact, multilamellar structure of myelin renders it difficult for antibodies to gain access to major encephalitogens such as MBP and PLP that are sequestered within this tightly packed compartment. As a consequence neither MBP nor PLP autoantibodies exhibit demyelinating activity *in vitro* ((Seil et al., 1973; Seil and Agrawal, 1980) and DNA vaccines encoding these autoantigens would not be expected to generate a pathogenic antibody response. The Ig-like domain of MOG, in contrast, is uniquely exposed at the outermost surface of the myelin sheath and oligodendrocyte (Kroepfl et al., 1996; Brunner et al., 1989). It is therefore readily accessible to bind antibodies present in the extracellular compartment and provides a target for antibody-mediated immune effector mechanisms (Kerlero de Rosbo et al., 1990; Piddlesden et al., 1993).

The pathogenicity of the circulating anti-MOG antibody response induced by MOG-DNA vaccination was demonstrated *in vitro.* First, these antibodies bound native MOG expressed on the surface of transfected cells (Figure 5). Binding of the native antigen is a prerequisite for antibodies to mediate a pathogenic response *in vivo*, but antibody-mediated demyelination involves the subsequent activation of complement-dependent and antibody-dependent cell-mediated cytotoxicity (ADCC) effector mechanisms (Linington et al., 1989; Piddlesden et al., 1991). Indeed, the *in vivo* demyelinating potential of MOG-specific antibodies is related to their ability to fix complement (Piddlesden et al., 1993). This was indeed the case for the antibody response induced by MOG-DNA vaccination as demonstrated by the MOG-specific antibodies' ability to lyse antigen-positive target cells in a complement and dose-dependent manner (Figure 18). Another proposed criterium for pathogenicity of MOG-specific antibodies is their epitope specificity. A set of demyelinating anti-MOG monoclonal antibodies did not recognise any of a panel of short overlapping MOG peptides, while non-demyelinating antibodies have linear specificities (Brehm et al., 1999). These findings suggest that pathogenic anti-MOG antibodies may be specific for conformation-dependent epitopes. The antibody response induced by MOG-DNA vaccination recognised strictly conformation-dependent epitopes (Figure 16), strengthening the hypothesis that these antibodies are pathogenic in vivo.

The pathogenicity of the autoantibody response induced by DNA vaccination is conditional, as MOG-DNA vaccinated mice show no signs of disease in the absence of an encephalitogenic challenge. Only when the blood-brain barrier is breached through the action of activated encephalitogenic T cells can the autoantibodies enter the CNS and mediate demyelination (Wekerle et al., 1994). The identity of the encephalitogen that initiates blood-brain barrier dysfunction is however irrelevant, since exacerbation can be seen following immunisation with either MOG or PLP, an unrelated encephalitogen. This parallels the situation seen in MOG-antibody transgenic mice where MOG-specific B cells and circulating autoantibodies fail to cause disease in the healthy organism, but demonstrate their pathogenic potential in combination with an encephalitogenic T cell response (Litzenburger et al., 1998).

The demonstration that the anti-MOG antibody response is pathogenic does not preclude that exacerbation of EAE following MOG-DNA vaccination could be mediated by a small population of MOG-specific T cells primed by DNA vaccination. These T cells could

then be rapidly activated by conventional immunisation and thereby be responsible for the early onset of disease seen following MOG-DNA vaccination. Such a mechanism has been suggested by Tsunoda et al. (Tsunoda et al., 1998) who demonstrate exacerbation of PLP-EAE following vaccination with DNA encoding a PLP peptide. The authors could show PLP-induced proliferation of $CD4^+$ splenocytes following DNA vaccination. Although in the current study no antigen-specific proliferation of T cells could be observed following DNA vaccination (Figure 10A), the generation of a population of primed encephalitogenic T cells below detection level cannot be excluded. These T cells cannot however be stimulated to induce EAE by repeated MOG-DNA vaccination, as 3 repeated MOG-DNA injections at days 0, 9 and 15 did not induce clinical signs of disease (data not shown). In addition, the mechanism by which a MOG-specific T cell response primed by DNA vaccination could exacerbate EAE induced with an unrelated encephalitogen is unclear.

Beside the antigen-specific effect of DNA vaccination, non-coding bacterial DNA can also influence the clinical course of EAE. Bacterial DNA contains immunostimulatory sequences that function as Th1 promoting adjuvants. These sequences stimulate immune responses to coadministered antigens by promoting IFNγ and IL-12 production, inducing a predominant IgG2a antibody response and generating cytotoxic T lymphocytes, all of which reflect a Th1 mediated immunity (Sato et al., 1996; Roman et al., 1997; Davis et al., 1998). The role of immunostimulatory sequences in EAE is somewhat contradictory. MBP-specific T cells activated in vitro by bacterial DNA induced EAE when transferred into otherwise resistant mice (Segal et al., 1997) and intramuscular injection of non-coding bacterial DNA increased PLP-specific IFNγ production and enhanced PLP-induced EAE (Tsunoda et al., 1998). The opposite is also true as non-coding plasmid DNA has been reported to suppress MBP-induced EAE (Boccaccio et al., 1999). In this study adoptively transferred T lymphocytes from plasmid-treated animals were less encephalitogenic than T cells from untreated animals. The authors suggest that suppression of EAE may be mediated by an increase in IFNγ production which can have a regulatory role in EAE (Voorthuis et al., 1990; Heremans et al., 1996). In the present study, although the expression vector used contains 11 immunostimulatory sequences, the effect on the clinical course EAE of vaccination with the control vector alone was minimal. While onset of MOG-EAE was similar in both pcDNA-vaccinated and non-vaccinated mice, a slight increase in clinical score was detected in pcDNA-vaccinated animals in the acute phase of disease (Figure 12). However, vaccination

with pcDNA had no significant effect on the clinical course of PLP-induced EAE (Figure 15A).

7.5. Strain-specific effect of MOG-DNA vaccination

An obvious approach to confirm that disease exacerbation following MOG-DNA vaccination is B cell-dependent was to perform the experiment in B cell-deficient mice (Kitamura et al., 1991). These were available on a C57BL/6 background, a strain susceptible to MOG-induced EAE. Upon challenge, no significant clinical difference was seen between MOG-DNA vaccinated B cell-deficient mice and their wild-type littermates. However, the C57BL/6 wild-type mice did not develop MOG-specific antibodies after MOG-DNA vaccination, prompting a comparative study in different mouse strains. This experiment showed that even strains resistant to MOG-induced EAE such as Balb/c and 129/sv develop an antibody response following MOG-DNA vaccination, but that the susceptible C57BL/6 strain had a complete absence of anti-MOG antibodies (Figure 21A, Table 3). Immunisation with MOG^{Igd} both in complete and incomplete Freund's adjuvant confirmed that C57BL/6 mice can mount an antibody response to MOG under strongly immunogenic conditions (Figure 22, Table 3), indicating that the absence of antibodies following DNA vaccination is not due to a defect in presentation or recognition of this antigen. Furthermore, the MHC haplotype does not seem to influence the response, as 129/sv mice that have the same MHC haplotype as C57BL/6 respond to MOG-DNA vaccination, albeit with lower antibody titres.

Strain	Antibody response		EAE susceptibility	MHC haplotype
	to MOG-DNA	to MOG^{Igd}		
SJL	+++	+++	+++	H-2s
Balb/c	+++	+++	-	H-2d
129/sv	++	++	-	H-2b
C57BL/6	-	++	++	H-2b

Table 3: Comparison of antibody responses and EAE susceptibility in different mouse strains
Comparison of antibody responses following vaccination with MOG-DNA or immunisation with recombinant MOG protein and susceptibility to EAE in different mouse strains.

A possible explanation is that MOG cDNA might not be expressed in C57BL/6 mice following DNA vaccination due to strain-specific variations in transcription or translation. This seems however unlikely, as this strain is generally known to be a good responder to DNA vaccination and to develop strong antibody responses to encoded antigens (Kuhrober et al., 1997; Wiest-Ladenburger et al., 1998). An antibody response to hepatitis B viral envelope proteins in C57BL/6 mice has also been reported after vaccination with the same vector (pcDNA3) used in this work (Chow et al., 1998). Alternatively, failure to mount an antibody response following MOG-DNA vaccination could be due to partial tolerance induced by the endogenous expression of MOG or of a homologous protein outside the CNS in C57BL/6 mice. This hypothesis is supported by the phenomenon seen in double-transgenic MOG Ig mice. These mice express both the gene-targeted Ig heavy chain and the conventional transgenic light chain of a MOG-specific monoclonal antibody (Litzenburger et al., submitted). Editing of the light chain in C57BL/6 mice suggests tolerisation by an antigen with homology to MOG expressed in the periphery. One straightforward experiment to test this hypothesis would be to perform MOG-DNA vaccination in MOG-knock-out mice on the C57BL/6 background. If tolerisation is due to expression of MOG in the periphery, MOG-deficient mice would not be tolerised and should mount an anti-MOG antibody response following vaccination. However, if tolerisation is induced by expression of another antigen in the periphery, identification of the antigen would involve extensive genotyping studies.

7.6. Conclusion and perspectives

In this study, rather than enhancing tolerance and decreasing susceptibility to EAE, MOG-DNA vaccination induced a Th1 associated autoantibody response that increased disease activity. This is in contrast to results obtained using DNA constructs encoding MBP or PLP peptides that suppress T cell responsiveness to the target autoantigen and have a marked beneficial effect on disease activity (Lobell et al., 1998; Ruiz et al., 1999). As yet, very little is known as to the mechanisms that induce either immunity or tolerance following DNA vaccination.

Repeated injection of small quantities of antigen can lead to the development of immunologic unresponsiveness (Liblau et al., 1997). As the amount of antigen produced by DNA vaccination is small, and expression can persist for weeks or months, vaccination with DNA might in theory induce tolerance rather than immunity. Interestingly, tolerance has

however rarely been observed following vaccination with DNA encoding viral or parasitic antigens (Donnelly et al., 1997), a phenomenon which may be related to the Th1 adjuvant effect of bacterial DNA. One exception is vaccination of neonates, which can occasionally induce tolerance. For example, vaccination with DNA encoding the circumsporozoite protein of the malaria parasite elicits protective immunity in adult mice but induces tolerance in 2-5 day old mice (Mor et al., 1996). In contrast, conventional immunisation of age-matched controls with recombinant protein did not result in tolerance but induced an immune response. Factors that may be associated with the development of neonatal tolerance are the age at immunisation, as susceptibility to tolerance induction to circumsporozoite protein disappeared in mice within one week of birth, and the amount of plasmid injected, as higher doses of plasmid were more tolerogenic (Ichino et al., 1999).

In adults, the outcome of DNA vaccination may depend on recognition of the encoded antigen as “self” or “non-self”. A preexisting tolerance to self antigens may be reinforced by DNA vaccination, whereas expression of foreign proteins such as viral or parasitic antigens in the context of DNA vaccination leads to immunity. As MOG is expressed exclusively in the CNS, the MOG-reactive T cell repertoire is not exposed to MOG in the periphery and tolerance is maintained by clonal ignorance. As a consequence the response to vaccination with MOG-DNA would be anticipated to resemble that seen following challenge with DNA coding for a truly “foreign” antigen. Intriguingly, this is indeed the case, since DNA vaccination with constructs encoding viral proteins induce a Th1-like response associated with a strong B cell component (Donnelly et al., 1997), a response similar to that seen following MOG-DNA vaccination.

Minor variations in experimental conditions may also radically influence the development of tolerance or immunity following DNA vaccination, as is demonstrated by two contradictory reports in which vaccination with DNA encoding the same autoantigen in similar experimental conditions either suppresses or enhances EAE (Tsunoda et al., 1998; Ruiz et al., 1999).

In practical terms this study demonstrates that vaccination with DNA encoding autoantigens should be considered with extreme caution, as the outcome is unpredictable. Certain strategies might however prove effective in restoring at least partial tolerance in

autoimmune diseases, in particular if suppression occurs at both the T and B cell level. One approach to suppressive DNA vaccination in Th1-mediated EAE could be to shift the antigen-specific response induced by DNA vaccination towards a Th2-like response. Indeed, several reports have shown that immune deviation therapy using antigen in other forms can suppress EAE (Racke et al., 1994; Kuchroo et al., 1995; Cua et al., 1995). This strategy may allow reinforcement of existing immunoregulatory networks without enhancing pathogenic Th1 responses. In addition, the antibody isotypes associated with a Th2-like response (IgG2a in mice, IgG4 in humans) poorly fix complement and may have only limited demyelinating effect. Although DNA vaccination usually induces a Th1-type response due to bacterial immunostimulatory sequences, skewing of the immune response towards Th2 can be achieved in several ways (Donnelly et al., 1997). DNA encoding a secreted rather than transmembrane or cytoplasmic antigen, coadministration of DNA encoding Th2-associated cytokines or intradermal injection of DNA with the use of a gene gun can all mediate a predominantly Th2 response (Cardoso et al., 1996; Pertmer et al., 1996; Feltquate et al., 1997; Boyle et al., 1997; Chow et al., 1998; Aberle et al., 1999; Lewis et al., 1999). Furthermore, vaccination with DNA encoding the variable region of a T cell receptor expressed on pathogenic T cells protected mice from EAE by reversing the autoimmune response from Th1 to Th2 (Waisman et al., 1996). However, skewing of the immune response towards Th2 is not always beneficial, as MBP-specific Th2 cells can in some cases actually cause EAE (Lafaille et al., 1997).

Since the main mechanism for exacerbation of EAE following vaccination with MOG-DNA appears to be via the pathogenic autoantibody response elicited, it may be possible to vaccinate with DNA constructs in which the domains recognised by the antibody response are disrupted while the T cell epitopes are left intact. As pathogenic anti-MOG antibodies are conformation-dependent, it should be fairly easy to design DNA constructs that preserve the primary T cells epitopes while disrupting the tertiary structure recognised by demyelinating antibodies. In this way, selective targeting of autoaggressive T cell responses might be achieved without stimulating a pathogenic antibody response.

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9. APPENDICES

9.1. Curriculum vitae

Carole Bourquin, born 01.05.1970 in Geneva, Switzerland

1988-1995	*University of Geneva, Faculty of Medicine* Medical studies
1991 and 1993	*University of Geneva, Faculty of Sciences* successfully passed Genetics and Molecular Biology of the Cell exams, required for Geneva MD-PhD program
3/1995	Swiss Diploma of Medicine
4/1995-9/1995	*University Hospital of Geneva, Clinic of Internal Medicine* Physician
11/1995-10/1996	*University Hospital of Geneva, Dpt. Of Clinical Pharmacology* (Prof. P. Dayer) Medical doctorate: "Cystein reduces the toxicity of bismuth on a membrane protein and on lymphocytes"
10/1996	Medical Doctor of the University of Geneva
1/1997-3/2000	*Max-Planck Institute for Neurobiology, Dpt of Neuroimmunology, Martinsried* (Prof. H. Wekerle) doctoral work in biology

Publications

A. Stefferl, U. Brehm, M. Storch, D. Lambracht-Washington, C. Bourquin, K. Wonigeit, H. Lassmann, C. Linington. Myelin Oligodendrocyte Glycoprotein induces experimental autoimmune encephalomyelitis in the „resistant" brown norway rat: disease susceptibility is determined by MHC and MHC-linked effects on the B cell response. *The Journal of Immunology, 1999, 163:40-49.*

C. Bourquin, A. Iglesias, T. Berger, H. Wekerle, C. Linington. Myelin oligodendrocyte glycoprotein-DNA vaccination induces antibody-mediated autoaggression in experimental autoimmune encephalitis. *European Journal of Immunology, 2000, 30:3663-3671.*

Abbreviations

8.18.C5	monoclonal anti-MOG antibody
Ag8	myeloma cell line X63-Ag8.6.5.3
Ag8/MOG	Ag8 cells transfected with pcDNA/MOG
AP	alkaline phosphatase
APC	antigen-presenting cell
bp	base pair
BSA	bovine serum albumine
cDNA	complementary DNA
CFA	complete Freund's adjuvant
CMV	cytomegalovirus
CNS	central nervous system
Con A	concanavalin A
cpm	counts per minute
CTL	cytotoxic T lymphocytes
DMEM	Dulbecco's modified Eagles' medium
DMSO	dimethylsulfoxyde
dNTP	desoxyribonucleotide
DTT	dithiothreitol
EAE	experimental autoimmune encephalitis
E. coli	Escherichia coli
EDTA	(Titriplex III) ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescent activated cell sorter
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
HE	hematoxylin-eosin
IFA	incomplete Freund's adjuvant
IFNγ	interferon gamma
Ig	Immunoglobulin
IL-4	interleukin 4
IPTG	isopropyl-1-thio-β-D-galactoside
ISS	immunostimulatory sequence
kb	kilobase pairs
kDa	kilodalton
KO	knock-out
MBP	myelin basic protein
MHC	major histocompatibility complex
MOG	myelin oligodendrocyte glycoprotein
MOG^{Igd}	MOG immunoglobulin-like domain
mRNA	messenger RNA
N.T.	not treated
OD	optical density
PCR	polymerase chain reaction
p.i.	post immunisation
PLP	myelin proteolipid protein
PPD	purified protein derivative
PTX	pertussis toxin

p.v.	post vaccination
rpm	revolutions per minute
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
s.c.	subcutaneous
SDS	sodium dodecylsulfate
TBE	Tris-boric EDTA buffer
Th1, Th2	T helper cell type 1 or 2
Tween 20	polyoxyethylene sorbitane monolaurate
WT	wild type