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Protracted, relapsing and demyelinating experimental autoimmune encephalomyelitis in DA rats immunized with syngeneic spinal cord and incomplete Freund's adjuvant

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Abstract

Experimental autoimmune encephalomyelitis (EAE) is a model for multiple sclerosis (MS). However, MS is a chronic, relapsing and demyelinating disease, whereas EAE in rats is typically a brief and monophasic disorder showing little demyelination. We demonstrate here that DA rats develop severe, protracted and relapsing EAE (SPR-EAE) after a subcutaneous immunization at the tail base with syngeneic spinal cord and incomplete Freund's adjuvant (IFA). The neurological deficits were accompanied by demyelinating inflammatory lesions in the spinal cord, with infiltrating T lymphocytes and perivascular deposition of immunoglobulins and complement. The induction of SPR-EAE was associated with humoral autoreactivity to myelin oligodendrocyte glycoprotein (MOG) and cellular autoreactivity to the rat myelin basic protein (MBP) peptides 69–87 and 87–101. These two peptides, as well as whole rat MBP, were encephalitogenic. In conclusion, we believe that the presently described demyelinating SPR-EAE represents a useful model for MS.

Keywords: Central nervous system; Myelin basic protein; T cells; Multiple sclerosis

1. Introduction

Experimental autoimmune encephalomyelitis (EAE), induced by immunization with central nervous antigen(s), is a widely used model for multiple sclerosis (MS) based on autoimmune, histopathological, genetic and clinical similarities (Raine, 1986; Olsson, 1992). The immunization elicits an autoimmune response towards myelin antigen(s) whereafter autoreactive T lymphocytes (Ben-Nun et al., 1981) migrate into the central nervous system (CNS) and, in possible concert with humoral autoreactivity (Linington et al., 1988; Myers et al., 1992), cause inflammation and neurological defects (Raine and Traugott, 1984; Lassman et al., 1991). The autoimmune response is restricted by class II genes within the major histocompatibility complex (MHC), but susceptibility to EAE is also determined by other non-defined genes (Happ et al., 1988; Matsumoto et al., 1990; Mustafa et al., 1993). Current knowledge of the processes leading to neurological defects and neuroinflammation have been extracted from a multitude of EAE forms induced in several species by various immunization protocols. However, no single EAE variant mirrors all features of MS and one major inadequacy in most rat EAE models is that they are typically brief and monophasic diseases whereas MS is chronic (Raine and Traugott, 1984; Lublin, 1985). Thus, neurological defects in rats typically develop and subside within a week, approximately 10 days after standard immunization procedures (injection into the footpads of an emulsion containing complete Freund's adjuvant (CFA) and xenogenic spinal cord, myelin basic protein (MBP) or a 68–88 MBP peptide). This brief disease course can be prolonged by pharmacological ma-

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nipulation, but these models have obvious limitations (Polman et al., 1988; Correale et al., 1991; Deguchi et al., 1991). Alternatively, the disease course may be prolonged by minor variations in the amount of injected spinal cord and/or mycobacteria (Panitch and Ciccione, 1981; Feurer et al., 1985), or by the use of additional adjuvants (Levine, 1986; Schorlemmer and Seiler, 1991). These chronic EAE models are rarely used, however, perhaps because it may be difficult to achieve a reproducible protracted disease course by the standard type of immunization protocols hitherto employed. Another explanation may be that LEW rats, the strain predominantly used in these and other EAE studies, are not prone to chronic EAE.

We have recently reported, briefly, that severe protracted and relapsing EAE (SPR-EAE) can be induced in the rarely used DA rat by a different and simplified immunization protocol, i.e. injection of syngeneic spinal cord and incomplete Freund's adjuvant (IFA) into the tail base (Lorentzen et al., 1995a).

Here we characterize SPR-EAE with regards to the following criteria: (1) reproducibility of the model; (2) susceptibility of LEW and (DAXLEW)F1 to SPR-EAE; (3) effects of variations in immunization parameters on the clinical course of EAE; (4) the humoral autoimmune response to myelin oligodendrocyte glycoprotein (MOG); (5) the cellular autoimmune response to different MBP peptides; (6) encephalitogenicity of rat MBP and the rat 69–87 and 87–101 MBP peptides; and finally (7) immunohistochemical and neuropathological features of the inflammation in the CNS at different time points after immunization with MBP peptide or spinal cord.

2. Materials and methods

2.1. Rats

Five different populations of DA rats were used. Rats bred and kept at the Department of Pathology in Uppsala and at the conventional animal department, Biomedical Center in Uppsala were originally obtained from Bantin and Kingman Ltd UK. Rats kept at Huddinge Hospital in Stockholm were purchased from Central Institute for Laboratory Animal Breeding, Hannover, Germany. Rats bred and kept at Karolinska Hospital and at the barrier animal department, Biomedical Center in Uppsala were originally obtained from the Central Institute for Laboratory Animal Breeding, Hannover, Germany. Rats were used in experiments aged 10–29 weeks, most rats being 13–17 weeks of age. All rats were provided food and water ad libitum.

2.2. Induction and clinical assessment of EAE

Antigens used for immunization were as follows; (1) spinal cord was obtained from DA rats or guinea pigs as stated in the text, tables and figures. The spinal cord was frozen, thawed and minced thoroughly with a spatula before immunization; (2) rat MBP was prepared according to a previously described method (Deibler et al., 1972) and dissolved in PBS (pH 7.4); (3) rat MBP peptides were dissolved in 1 mM acetic acid to a concentration of 2.5 mg/ml. Rats were immunized by one subcutaneous injection (just under the skin) into the dorsal base of the tail with 200 μl emulsion prepared from one part IFA (Difco, Detroit, MI) and one part antigen (volume/weight, i.e. 100 μl IFA/100 mg of whole spinal cord or 100 mg (= 100 μl) solution containing MBP or MBP peptides). In some experiments, IFA was complemented with 20 mg/ml of *Mycobacterium tuberculosis*, strain 37 RA (Difco) (= complete Freund's adjuvant, CFA) or substituted with PBS, i.e. no adjuvant, or with a 1 mg/ml suspension of particulate β-glucan adjuvant in PBS (Optivant™, TSI, Worcester, MA). The emulsion was prepared by tituration with a gas-tight glass syringe and a needle, 1.2 mm diameter. Rats were regularly weighed and examined for clinical signs of EAE. A three-graded scale was used to assess clinical severity: 0, no illness; 1, 'mild EAE', i.e. flaccid tail (hanging); 2, 'moderate' EAE, i.e. moderate inferior paraparesis (unsteady walk); 3, 'severe EAE', i.e. severe paraparesis (unable to walk, lying), some of these rats died or were killed when moribund and are referred to as suffering from lethal EAE. The term SPR-EAE applies to rats in which two or more attacks of severe EAE occurred and in which severe EAE had been observed over a period of seven days or more.

2.3. Histology, immunohistology and in situ hybridization

Animals were sacrificed at various stages of SPR-EAE (day 10, $n = 3$; day 12, $n = 2$; day 13, $n = 2$; day 23, $n = 3$; day 24, $n = 2$; day 32, $n = 8$) and perfused with cold 4% paraformaldehyde in 0.1 M phosphate buffer via the aorta. The brains and spinal cord were dissected and postfixed in the same fixative overnight at 4°C, washed in PBS and routinely embedded in paraffin. Paraffin sections were stained with Haematoxylin–Eosin, Luxol fast blue and Bielschowsky silver impregnation to assess inflammation, demyelination and axonal pathology, respectively. The number of perivascular inflammatory infiltrates was determined for each animal on an average of 10 complete cross-sections of spinal cord, covering a total area of spinal cord tissue of 30 mm^2. The extent of axonal loss was evaluated by comparing density of axons in the lesions with axonal density in perilesional areas. In the adjacent serial sections immunohistochemistry was performed with antibodies (Ab) against the following targets: macrophages/activated microglia (ED1; Serotec, Oxford, UK), T cells (W3/13; Seralab, Sussex, UK), C9 complement component (Piddlesden et al., 1994), rat immunoglobulin (biotinylated α-rat, Amersham, Buckinghamshire, UK), MOG (Piddlesden et al., 1993), 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase, Affinity Research Products,

Ilkeston, UK) and glial fibrillary acidic protein (GFAP, Boehringer, Mannheim, Germany). Bound primary antibody was detected with a biotin–avidin technique as previously described in detail (Vass et al., 1986). Control sections were incubated in the absence of primary Ab or with non-immune rabbit serum. In situ hybridization (ISH) with digoxigenin-labelled riboprobes specific for proteolipid protein (PLP, Coleman et al., 1982) was performed as described in detail (Breitschopf et al., 1992). The specificity of the reaction was controlled by comparing hybridization with antisense and sense riboprobes. ISH was followed by immunohistochemistry for PLP (Gunn et al., 1990) with an alkaline phosphatase/antialkaline phosphatase technique (Vass et al., 1989).

A detailed analysis of cells infiltrating the spinal cord during SPR-EAE was performed as follows: rats were sacrificed by ether inhalation. An approximately 15 mm long segment of the lumbar spinal cord was snap frozen in liquid nitrogen. Cryostat sections were melted onto microscopic slides and exposed to appropriate dilutions of the following monoclonal antibodies (mAbs) to rat cell surface antigens: OX19, anti-CD5 (Dallman et al., 1991); OX8, anti-CD8 (Brideau et al., 1980); W3/25, anti-CD4 (Williams et al., 1987); OX39, anti-IL-2R (Sedgwick et al., 1987); OX33, anti-B cells (Woolett et al., 1985); OX6, anti-MHC class II (McMaster and Williams, 1979); and OX18, anti-MCH class I (Fukomoto et al., 1982). The mAbs were purified from culture supernatants (Holmdahl et al., 1985) of hybridomas originally obtained from Dr Alan Williams (Oxford, UK), except for OX33, OX39 and OX18 which were purchased from Seralab (Crawley-Down, UK). The avidin-biotin peroxidase method was used for staining (ABC vectastain Elite kit, Vector lab, Burlingame, CA). Omission of the primary mAb served as negative control. Sections of peripheral lymphoid tissues served as positive controls.

2.4. Measurement of humoral autoreactivity to MOG

Blood was collected at the times stated in the text; either from the tail vein or by cardiac puncture immediately before perfusion. After clotting at 4°C, serum was collected by centrifugation and stored at −20°C. Enzyme-linked immunosorbent assay (ELISA) was performed using polystyrene 96-well ELISA plates (Costar, Bodenheim, Germany) coated overnight at 4°C with 10 μg/ml of recombinant MOG (the extracellular domain) in 100 mM borate saline buffer pH 8.6. The plates were washed with PBS pH 7.4/Tween 20 for 2 h at 37°C. After extensive washing with PBS/Tween, 100 μl of serum diluted in PBS/Tween was added to each well and incubated for a further hour at 37°C. Antibody bound to the plates was then detected using 100 μl of peroxidase-conjugated anti-rat IgG antibody (1:5000 in PBS, Dianova, Hamburg, Germany) which was added to each well after washing with PBS/Tween. Plates were developed after 1 h at 37°C with *o*-phenylenediamine dihydrochloride (SIGMA, GmbH, München, Germany), the reaction was stopped with 3 M HCl and the optical density determined at 490 nm.

2.5. MBP peptides

Eight different MBP peptides were used in assays of T cell autoreactivity. Two of these peptides were additionally used for immunization and had the amino acid sequence of rat MBP described by Martensson (1984). The 69–87 peptide, YGSLPQKSQRTQDENP-single letter code and the 87–101 peptide, PVVHFFKNIVTPRTP were synthesized using tBoc and Fmoc chemistry, respectively, in a ABI430A peptide synthesizer (Applied Biosystems, CA). After deprotection and cleavage the peptides were purified by reverse phase chromatography and analyzed by mass-spectrometry (Biolon Nordic AB, Uppsala, Sweden) showing the expected masses as major components in the spectra. These two peptides were synthesized and provided by Åke Engström, BMC, Uppsala. The other six MBP peptides, similarly synthesized, were selected on the basis of their encephalitogenicity in other strains or species: 1–11, ASQKRPSQRHGSKYLTAST (Urban et al., 1989); 68–88, **AARTTHYGSLPQKSQRSQDENPVHF** (Vandenbark et al., 1985); 89–101, VHFFKNIVTPRTP (Sakai et al., 1988); 110–128, GLSLSRFSWGAEGQKP-GFG Ben-Nun et al., 1981); 118–129 + 141–150, VTPR-TPPPSQGKGAEGQKPGFG; and 148–165, TLSKIFKLG-GRDSRSGSP (Karkhanis et al., 1975). Five of these six peptides correspond to the rat MBP sequence, while the 68–88 peptide differs at the positions printed in bold.

2.6. T cell autoreactivity to rat MBP peptides measured by antigen-induced interferon-γ secretion and proliferation

Rats were killed at 14 days p.i., regional lymph nodes were dissected and mononuclear cells were obtained by grinding the lymph nodes through a wire mesh. Cells were washed three times in culture medium consisting of Iscove's modification of DMEM (Flow, Irvine UK), supplemented with 2 mM glutamine (Flow), 50 IU penicillin, 60 μg streptomycin (Gibco, Paisly, UK) and 5% heat-inactivated fetal calf serum (Gibco). For evaluation of cells secreting interferon-γ (IFN-γ) upon antigen stimulation, an immunospot assay that enables detection of individual IFN-γ producing cells (Czerkinsky et al., 1988; Kabilan et al., 1990) and adapted for rat IFN-γ (Mustafa et al., 1991) was used. Briefly, 96 well nitrocellulose bottomed microtiter plates (Microtiter-HAM, Millipore Co., Bedford, MA) were coated with 100 μl aliquots of the mouse mAb anti-rat IFN-γ DB 1 (Van der Meide et al., 1986), a generous gift from Dr Peter H. Van der Meide, TNO Medical Biological Laboratory Rijswijk, Netherlands. Duplicate aliquots of 200 μl lymph node cell suspension

containing 5×10^6 MNC/ml were then applied. MBP peptides or concanavalin A (Flow) were added to obtain a final concentration in the medium of 10 and 5 μg/ml, respectively. These concentrations were determined to be optimal in preliminary experiments for this assay and for the proliferation assay described below. Other duplicate wells received no antigen to serve as background controls. Plates were incubated at 37°C, 7% CO_2 and a humid atmosphere for 48 h, then cells were discarded and plates washed. Secreted and bound IFN-γ was visualized by a rabbit antiserum to rat IFN-γ (Van der Meide et al., 1986) and secondary immunochemicals (ABC Vectastain elite kit; Vector lab) resulting in red-brown spots corresponding to individual cells that had secreted the cytokine. To study the antigen-induced proliferation of mononuclear cells, triplicates of 200 μl aliquots of lymph node cell suspension containing 3×10^6 cells/ml were applied in round-bottomed 96 well microtiter plates (Nunc). Different MBP peptides and lectins were added as described above. After 60 h culture, the cells were pulsed for 10 h with [^{3}H]methylthymidine (10 μl, 100 μCi/ml, Amersham, Little Chalfront UK) and harvested. Thymidine incorporation was assessed in a β-scintillation counter.

3. Results

3.1. SPR-EAE in DA rats immunized subcutaneously at the tail base with rat spinal cord and IFA

Immunizations were performed on nine different occasions and altogether on 60 male and female DA rats in five different animal departments. The susceptibility to EAE was high (80%) and the median day of the first clinical signs was 10 days post-immunization.

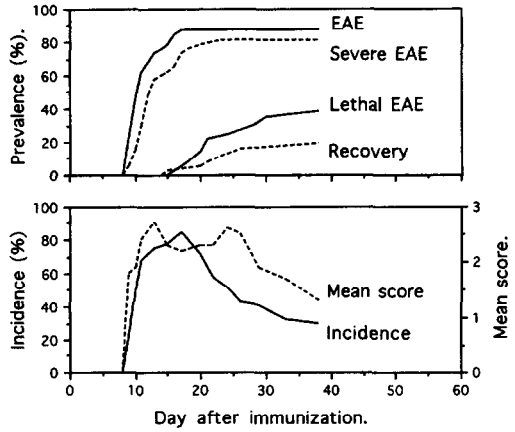

Fig. 1. Protracted EAE in DA rats. Incidence and mean score data on the development of EAE in animals immunized subcutaneously at the tail base with rat spinal cord emulsified in IFA (experiments 1–2 and 4–9a in Table 1). The incidence was calculated as the number of diseased rats per number of living rats at each time-point. The mean scores were only calculated on diseased rats.

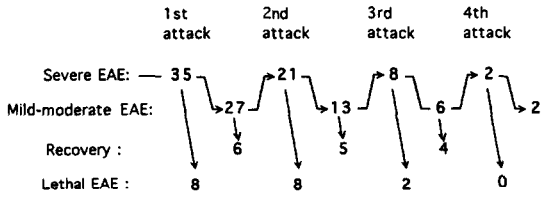

Fig. 2. Relapsing EAE in DA rats. The clinical outcome of 35 animals with severe EAE (score 3) following subcutaneous immunization at the tail base with rat spinal cord emulsified in IFA (experiments 1–2 and 4–9a in Table 1). Rats with severe EAE either died or were killed at a moribund state (lethal EAE), or improved clinically to mild or moderate EAE (score 1 or 2, respectively). From this condition they either recovered completely from clinical signs of neurological deficits (score 0) or relapsed back to severe EAE.

The disease course was strikingly prolonged compared to acute EAE in rats, in which severe EAE lasts only a few days. Thus, in our experiments, some DA rats had severe neurological deficits over a period of 30 days. This protracted disease course is evident from the scores of neurological defects and weight data for individual animals (Fig. 3) or median score and incidence data for all animals (Fig. 1). The majority of animals (68%) developed a severe form of EAE with complete paraparesis, wasting and often bladder incontinence. Most of these (69%; 45% of all rats) had a protracted disease (defined as observations of severe EAE 7 days or more apart) and the clinical course (Fig. 2) usually included 1–3 relapses of severe EAE (63%; 42% of all rats). The duration of severe EAE attacks in individual animals was unpredictable, ranging from 1–13 days and lethal EAE often occurred (51%; 34% of all rats) in one of the first three attacks. SPR-EAE occurred in 60% of rats with severe EAE (40% of all rats) but this figure is 81% when calculated on rats surviving severe EAE for 7 days or more, e.g. long enough to fulfil the criteria set for SPR-EAE. There was a tendency towards recovery/improvement of neurological deficits in surviving rats, which is why we have chosen the term 'protracted' rather than 'chronic encephalomyelitis'. SPR-EAE developed in all

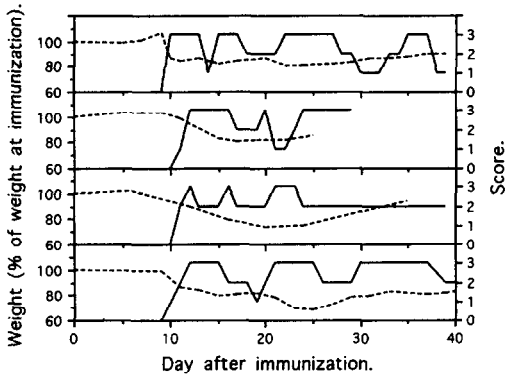

Fig. 3. Clinical course with respect to changes in body weight and scores of neurological defects in four selected individual DA rats immunized subcutaneously at the tail base with rat spinal cord emulsified in IFA (selected from experiments 2, 4, 6 and 7 in Table 1).

Table 1
EAE induced with spinal cord (SC) in different experimental situations

Immunization parameters					Clinical data					
Experiment number and location [a]	Strain	Sex [b] and number of rats	Injection site [c]	Origin of SC and type of adjuvant	Prevalence (%) of				Median	
					EAE	Severe EAE	SPR-EAE	Lethal EAE	Day of EAE onset	Max. weight loss [d] (%)
1, DP	DA	4 f.	Tail base, s.c.	Rat, IFA	100	100	75	50	13	–
2, DP	DA	7 f.	Tail base, s.c.	Rat, IFA	86	71	29	67	10	23
3, DP	DA	7 f.	Tail base, s.c.	Rat, IFA	100	86	n.a. [e]	n.a. [e]	10	n.a. [e]
4, DP	DA	6 m.	Tail base, s.c.	Rat, IFA	83	83	17	33	11	20
5 [f], BMC(c)	DA	5 f.	Tail base, s.c.	Rat, IFA	80	80	40	40	10	19
6, DP	DA	5 f.	Tail base, s.c.	Rat, IFA	60	60	20	20	13	10
7, HH	DA	10 m.	Tail base, s.c.	Rat, IFA	100	90	80	20	10	19
8a, KH	DA	6 m	Tail base, s.c.	Rat, IFA	100	50	50	50	10	10
9a, BMC(b)	DA	10 m	Tail base, s.c.	Rat, IFA	40	20	10	10	12	16
1–9a	**DA**	**60**	**Tail base, s.c.**	**Rat, IFA**	**80**	**68**	**40**	**30**	**10**	**18**
10	LEW	6 f	Tail base, s.c.	Rat, IFA	50	33	33	0	12	17
11	(DAXLEW)F1	4f	Tail base, s.c.	Rat, IFA	50	50	25	25	9	20
8b, KH	DA	6 m	Tail base, i.d.	Rat, IFA	100	100	17	100	9	14
9b,BMC(b)	DA	10 m	Tail base, s.c.	GP IFA	100	90	20	10	13	20
9c,BMC(b)	DA	10 m	Tail base, s.c.	Rat, CFA	100	100	40	50	11	28
9d,BMC(b)	DA	10 m	Tail base, s.c.	GP,CFA	90	90	40	50	11	24
9e,BMC(b)	DA	10 m	Footpads	GP,CFA	100	100	30	60	12	26
12, DP	DA	5 f.	Footpads	Rat, IFA	100	100	80	20	10	18
13, DP	DA	5 f.	Tail base, s.c.	Rat,-(PBS)	20	20	0	0	23	7
14, BMC(c)	DA	5 f.	Tail base, s.c.	Rat, β-glucan	100	100	0	80	10	16

[a] Abbrevations for location: DP, Department of Pathology in Uppsala; BMC(b), Biomedical Center in Uppsala, barrier animal housing; BMC(c), Biomedical Center, conventional animal housing; KH, Karolinska Hospital; HH, Huddinge Hospital.
[b] Sex: f, female; m, male.
[c] Injection site: s.c., subcutaneously; i.d., intradermally.
[d] Median maximal weight loss of diseased rats, compared to the weight at the day of immunization.
[e] n.a., not applicable, the experiment was terminated 20 days after immunization.
[f] Half the usual amount of SC was used.

Table 2
The reactivity of draining lymph node cells to MBP peptides in DA rats immunized with rat spinal cord emulsified in IFA

Antigen (Ag) added to culture	Analyzed cell reactivity							
	IFN-γ secretion [a]				Proliferation [b]			
	n	Mean	S.E.M.	*P* [c]	*n*	Mean	S.E.M.	*P*
ConA	14	274	33.4	< 0.001	13	189414	8056	< 0.001
Background (no Ag)	15	19	5.3		13	4657	623	
MBP peptides								
69–87	12	37	8.9	0.01	11	3791	404	0.52
87–101	12	31	7.6	0.06	11	4016	429	0.72
1–11	15	23	6.7	0.50	n.d. [e]	n.d.	n.d.	n.d.
GP [d] 68–88	15	37	7.9	0.03	13	5024	610	0.64
89–101	15	31	7.5	0.08	13	4379	539	0.79
110–128	15	24	6.7	0.30	13	4403	509	0.87
118–129 + 141–150	14	18	5.4	0.80	n.d.	n.d.	n.d.	n.d.
148–165	15	24	6.4	0.20	13	4529	541	1.00

DA rats were immunized subcutaneously at the base of the tail with rat spinal cord emulsified in IFA, 14 days before the experiments.
[a] Analyzed with an immunospot assay; data shown represent numbers of IFN-γ-secreting cells, spots, per 10^6 plated mononuclear cells. Compare numbers induced by added Ag to background controls (no Ag).
[b] Measured by [^{3}H]thymidine uptake.
[c] *P*-values were obtained comparing cultures receiving Ag with cultures receiving no Ag and were calculated by the non-parametric Mann-Whitney test.
[d] GP, guinea pig.
[e] n.d., not done.

Table 3
Development of EAE in DA rats after immunization with rat MBP or rat MBP peptides emulsified in IFA

Immunization parameters		Clinical data						
Rat auto-antigen	Sex [a] and number of rats	Prevalence (%) of immunized rats with					Day of EAE onset	Median max. weight loss [b] (%)
		EAE	Mild EAE	Moderate EAE	Severe EAE	SPR-EAE		
MBP	5 m. [c]	80	20	20	40	0	9–13	8
	5 f. [d]	100	0	40	60	20	11–14	10
	5 f. [e]	100	0	0	100	20	10–12	12
MBP69–87 peptide	6 f.	100	50	17	33	0	12–17	4
	7 m.	86	57	29	0	0	13–16	5
MBP87–101 peptide	6 f.	100	50	33	17	0	12–17	4
	7 m.	58	29	29	0	0	13–16	7

DA rats were immunized subcutaneously, at the base of the tail, with MBP or MBP peptides emulsified in IFA.
[a] f. = female, m. = male.
[b] in diseased rats, compared to weight at the day of immunization.
[c] 130 μg MBP/rat.
[d] 180 μg MBP/rat, individual disease courses shown in Fig. 4.
[e] 130 μg MBP/rat, intradermal immunization.

experiments (Table 1, exp. 1–9a) demonstrating that the model is reproducible. To examine if SPR-EAE was genetically restricted to DA rats, we immunized LEW and (DAXLEW)F1 rats with rat spinal cord and IFA. In these experiments, 2/6 LEW and 2/4 (DAXLEW)F1 rats developed SPR-EAE (Table 1, exp. 10–11).

3.2. Effects of the immunization site, origin of spinal cord and type of adjuvant on the clinical course of EAE in DA rats

We investigated the importance of different immunization variables for the induction of SPR-EAE in the DA rat (Table 1). In a series of experiments (exp. 9a–9e and 12) we observed that SPR-EAE could be induced with any combination of the following variables; rat/guinea pig spinal cord, IFA/CFA and tail base/footpad immunization. Notably, the EAE severity in the 10 animals immunized at the tail base with rat spinal cord in IFA was unusually low (exp. 9a). We also investigated the effect of intradermal instead of subcutaneous immunization at the tail base and observed that intradermal immunization caused a more severe, lethal EAE and a lower incidence of SPR-EAE (exp. 8a,b).

That the adjuvant used can affect both susceptibility and disease course is evident from the experiments in which only a brief or mild EAE followed when no adjuvant was added to the spinal cord inoculum (exp. 13), while the disease was severe and lethal when β-glucan was used as an adjuvant (exp. 14).

3.3. MBP autoreactivity in DA rats immunized with rat spinal cord in IFA

To define potentially encephalitogenic proteins and determinants in the antigenically complex spinal cord, we focused on MBP since guinea pig MBP is encephalitogenic in DA rats (Mostarica-Stojković et al., 1982). Altogether, 15 rats in four separate experiments, were immunized with rat spinal cord emulsified in IFA and 14 days p.i., the in vitro reactivities of mononuclear cells to a panel of MBP peptides were examined (Table 2). Two MBP peptides, 69–87 and 87–101, induced an increase in numbers of IFN-γ producing cells. The proliferative response was not significantly elevated above background levels with any of the MBP peptides used.

3.4. Encephalitogenicity of rat MBP and the MBP 69–87 and 87–101 peptides

Altogether, 15 DA rats were immunized subcutaneously at the tail base with 130–180 μg/rat of rat MBP in IFA.

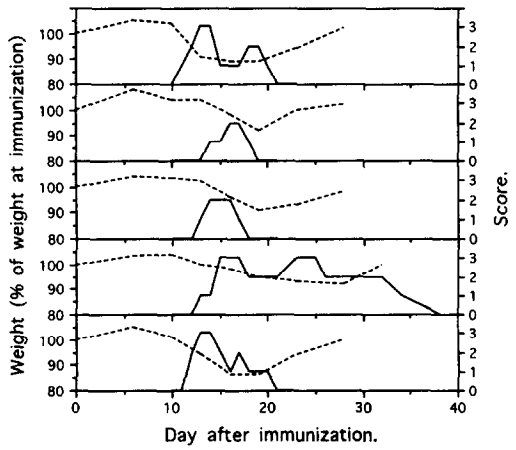

Fig. 4. Clinical course with respect to changes in body weight and scores of neurological defects in five female DA rats immunized subcutaneously at the tail base with rat MBP emulsified in IFA (these data correspond to the second MBP experiment in Table 3).

Fourteen rats developed EAE, 10/15 developed severe EAE and 2/10 developed SPR-EAE (Table 3). Disease courses of individual rats are depicted in Fig. 4. Using lower amounts of rat MBP in male rats, severe EAE became less frequent but could be induced with as little as 33 μg rat MBP/rat (data not shown). We next examined the encephalitogenicity of the 69–87 and 87–101 rat MBP peptides. Rats were immunized with each of the two peptides emulsified in IFA (Table 3). Neurological defects developed in a high proportion of rats and paresis ensued in 5/13 of the immunized animals, mostly in females. A relapsing and prolonged disease course was evident in some rats. (Fig. 5).

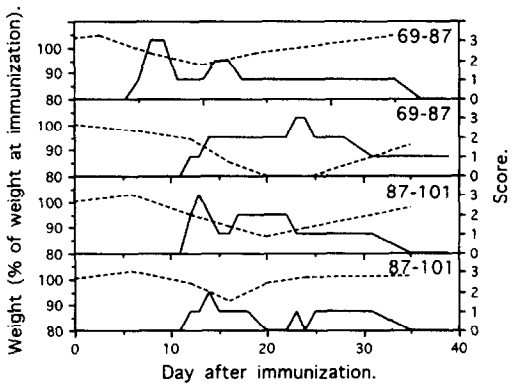

Fig. 5. The clinical course with respect to changes in body weight and scores of neurological defects in four selected DA rats immunized subcutaneously at the tail base with rat MBP 68–89 or 89–101 peptide emulsified in IFA.

3.5. The phenotypes of cells infiltrating the CNS of DA rats immunized with rat spinal cord or rat MBP peptides in IFA

We performed an immunohistochemical evaluation of immunized animals to examine if the observed neurological defects were accompanied by inflammation in the CNS. Both spinal cord and peptide immunized rats exhibited perivascular inflammation with infiltration of T cells

Table 4
Survey over immunohistochemical stainings of cryosectioned spinal cords from DA rats with EAE

Rat auto-antigen/ sampling time/rat designations	MHC antigens		Pan T cells CD5 (OX19)	Th and MΦ CD4 (W3/25)	T c/s CD8 (OX8)	Activated T cells IL-2R (OX39)	B cells (OX33)
	Class I (OX18) [a]	Class II (OX6)					
Spinal cord							
Day 14 p.i.							
3I	+ + +	+ + +	+	+ + +	+ +	+ + +	5
3II	+	+	0	+	+	0	0
3III	+ +	+ +	+ +	+ +	+ +	+	0
3IV	+ +	+ +	+	+	+	+	0
3V	+ +	+ +	+	+ +	+ +	+	0
Day 20 p.i.							
1I	+ +	+ +	+	+	+ +	+ +	0
1III	+ +	+ +	+	+ +	+	+	0
2I	+ +	+ +	+	+	+ +	+	1
2II	+ +	+ +	+	+ +	+ +	+ +	0
3I	+ + +	+ + +	+ +	+ +	+ + +	+ +	0
3II	+ + +	+ + +	+ +	+ +	+ +	+ +	0
3III	+ + +	+ + +	+	+ +	+ + +	+ +	0
MBP69–87							
Day 14 p.i.							
I	+ + +	+ + +	+ +	+ +	+ +	+	0
II	+ + +	+ + +	+	+ +	+	+	3
III	+ + +	+ + +	+	+ +	+	+	0
IV	+ +	+ +	+	+ +	+	+	0
V	+ + +	+ + +	0	+ +	+ +	+	0
MBP 87–10							
Day 14 p.i.							
I	+ + +	+ + +	+	+ +	+	+	0
II	+ + +	+ + +	+	+ + +	+ +	+	5
III	+ + +	+ + +	+	+ + +	+ +	+	5
IV	+ + +	+ + +	+ +	+ +	+ +	+	0
V	+ +	+ +	+	+ +	+	+	0

Spinal cords were taken from DA rats immunized subcutaneously, at the base of the tail, with rat spinal cord or rat MBP peptides emulsified in IFA. Data from individual rats are shown. The numbers of stained cells per spinal cord cryosection were graded semiquantitatively: +, 1–99; + +, 100–999; + + + > 1000.

[a] Antibodies for labelling are given in brackets.

and abundant MHC class I and II expression, 14 days and 20 days post-immunization (Table 4). Many of the infiltrating T cells were activated (IL-2R$^+$) and both CD8$^+$ and CD4$^+$ T cells were present.

3.6. Neuropathology of DA rats immunized with rat spinal cord in IFA

The pathology of SPR-EAE was characterized by perivenous inflammation in association with a variable extent of active or inactive demyelination. In Fig. 6 we demonstrate that actively demyelinating lesions mainly occurred in the early stages of SPR-EAE (day 10–13). In the late stages (day 23–32) demyelinating activity was rare, although one animal exhibited extensive actively demyelinating plaques in the very late stage (day 32; Fig. 6; Table 5). Active lesions were characterized by destruction of myelin and oligodendrocytes (Fig. 6a), abundant infiltration of macrophages (Fig. 6c) and lymphocytes, as well as granulocytes (Fig. 6d), massive perivascular granular deposition of complement component C9 (Fig. 6e) and precipitation of IgG, reflecting blood–brain barrier damage. Axons were either preserved or only slightly reduced (Fig. 6b). Chronic inactive plaques revealed extensive confluent demyelination (Fig. 7) associated with reduction and loss of oligodendrocytes, as visualized by immunocytochemistry for cyclic-nucleotide-phosphodiesterase (CNPase) or by in situ hybridization for PLP mRNA. Inflammatory infiltrates mainly contained macrophages, little or no T cells and no granulocytes. Only traces of IgG and C9 were present in the lesions, mostly intracellularly in perivascular macrophages. Axons were lost in chronic lesions to a variable degree (Table 5). Most extensive reduction of axons was present in the dorsal column of the spinal cord. In general, the lesions were largest in the spinal cord, smaller in the cerebellar white matter, medulla oblongata and mesencephalon. Very little pathological alterations were observed in forebrain regions. Inflammatory infiltrates consisting of macrophages and lymphocytes were also evident in the peripheral nervous system (PNS) exam-

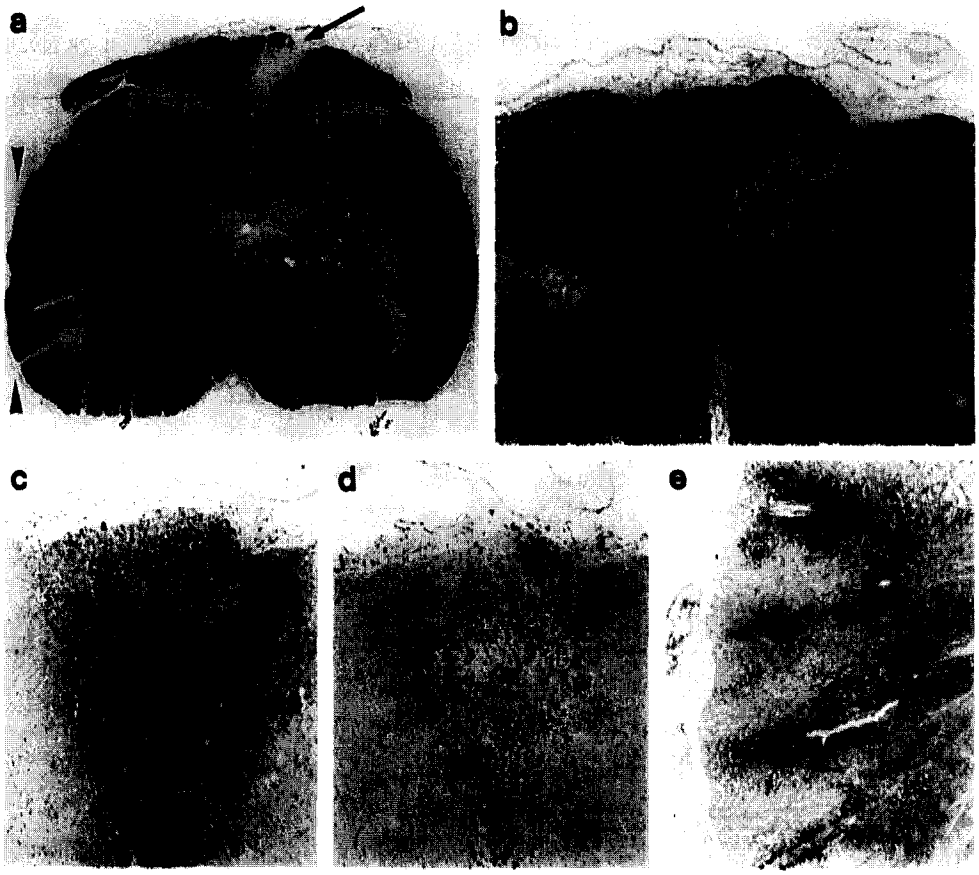

Fig. 6. Representative neuropathology of spinal cord (32 days after immunization) with early active demyelination in DA rats immunized subcutaneously at the tail base with rat spinal cord emulsified in IFA. Serial cross sections were analyzed with different stains and markers. (a) Luxol fast blue myelin stain. Multiple areas of perivenous demyelination with confluent demyelinated lesions in the dorsal column. The arrow indicates the lesion shown in higher magnification in Fig. 1b–d, the arrowheads indicate the location of Fig. 1e (×30) (b–d) Adjacent sections stained with Bielschowsky silver impregnation (b; ×95) and immunostained for macrophages with mAb ED1 (c; ×95) and for T cells with mAb W3/13 (d; ×95). (b) Only slight reduction of axonal density. (c,d) Inflammatory infiltrates are composed of macrophages and W3/13-positive lymphocytes and granulocytes. (e) Immunostaining for complement component C9 deposition in adjacent section (×75). Demyelination is accompanied by massive perivascular granular deposition of C9.

Fig. 7. Representative neuropathology of spinal cord (24 days after immunization) with large confluent inactive demyelinated lesions in DA rats immunized subcutaneously at the tail base with rat spinal cord emulsified in IFA. Luxol fast blue myelin stain (×40).

ined (spinal nerve roots) but demyelination was absent. Non-immunized rats served as controls, they did not exhibit any pathology.

Table 6
Humoral autoreactivity to MOG in DA rats immunized with rat spinal cord (SC) emulsified in IFA

Strain	Inoculum	EAE score [a]	OD	SD
DA	SC + IFA	1	0.689	0.038
DA	SC + IFA	3	0.184	0.010
DA	SC + IFA	1	0.239	0.007
DA	SC + IFA	0	0.214	0.074
DA	SC + IFA	2	0.252	0.029
DA	SC + IFA	3	0.913	0.034
DA	SC + IFA	0	0.187	0.022
DA	SC + IFA	0	0.148	0.004
DA	–	0	0.019	0.012
DA	–	0	0.010	0.010
LEW	MOG + CFA		1.736	0.068
LEW	MOG + CFA		1.459	0.111
LEW	MOG + CFA		1.375	0.088

The DA rats were immunized subcutaneously, at the tail base with rat spinal cord emulsified in IFA. Sera, diluted 1/200, were tested for reactivity to MOG by standard ELISA procedure. The OD values are corrected for the serum background (OD = 0.072, SD = 0.037) and are the means of quadruplicate values. The sera from LEW rats were taken 14 days after immunization. The sera from DA rats were taken 32 days after immunization from rats which had previously developed severe EAE (score 3).

[a] The EAE score 32 days after immunization.

Table 5
Neuropathology in DA rats immunized with rat spinal cord emulsified in IFA

Day after immunization	EAE score	Inflammatory incidence	Demyelination		IgG	C9	Axonal pathology
10	3	7.1	++	A = I	++	++	+
10	3	3.5	++	A > I	++	++	+
10	3	5.0	+	I > A	+	+	+
12	3	7.4	++	I > A	+	++	+
12	3	1.4	+	I > A	±	+	±
13	3	3.1	++	I > A	+	++	+
13	3	7.6	++	I > A	+	++	+
23	1	0.5	±	I	±	±	–
23	0	3.3	±	I	±	±	±
23	3	1.7	+	I	±	±	+
24	3	10.5	++	I ≫ A	+	+	+
24	3	2.0	+++	I ≫ A	±	±	+
32	1	0.3	++	I ≫ A	±	±	++
32	3	0.9	+++	I ≫ A	±	±	++
32	1	0.3	+++	I	±	±	++
32	0	0.2	±	I	±	±	±
32	2	1.4	+++	I ≫ A	±	±	++
32	3	3.1	+++	A	++	++	±
32	0	0.1	±	±	±	±	±
32	0	1.8	±	±	+	+	±

DA rats were immunized subcutaneously, at the base of the tail, with rat spinal cord emulsified in IFA. Assessment of inflammation, demyelination and axonal pathology performed on paraffin sections of spinal cord. Inflammatory incidence: an average number of perivascular inflammatory infiltrates/10 complete cross-sections.

Demyelination: A, active; I, inactive; ±, occasional perivascular demyelination; +, small rims of perivascular and/or subpial demyelination; ++, marked perivascular and subpial demyelination; +++, large confluent demyelination. IgG: ±, traces of IgG precipitation in lesions, mostly intracellularly; +, in addition, also marked perivascular leakage in some lesions; ++, massive perivascular/parenchymal leakage. C9: ±, traces of C9 in the lesion, mostly intracellularly; +, some perivascular lesions with granular deposition of C9; ++, massive perivascular granular deposition of C9. Axonal pathology: ±, slight reduction of axonal density (< 10%); +, reduction of axonal density to 90–50% of normal values; ++, reduction of axonal density to 50–0% of normal values.

3.7. Humoral autoreactivity to MOG in DA rats immunized with rat spinal cord in IFA

In Table 6 we demonstrate that autoantibodies to the extracellular domain of MOG develop in DA rats immunized with rat spinal cord in IFA.

4. Discussion

Chronic, relapsing and demyelinating variants of EAE can be induced by active immunization in different species and strains including guinea pigs (Stone and Lerner, 1965), rabbits (Prineas et al., 1969), mice (Brown et al., 1982) and, as described here, also in rats.

We demonstrate that DA rats develop severe protracted and relapsing EAE (SPR-EAE) after immunization with rat spinal cord together with IFA at the tail base. Neurological defects are accompanied by perivascular demyelinating inflammation in the spinal cord with complement deposition, infiltrating T lymphocytes and MHC class I and II expressing cells. Humoral autoreactivity to MOG is evident in these animals and mononuclear cells from lymph nodes draining the immunization site react preferentially, in terms of antigen-induced IFN-γ secretion, to the 69–87 and 87–101 rat MBP peptides among several MBP epitopes. These two peptides, as well as whole rat MBP, are encephalitogenic when used for immunization together with IFA.

The present SPR-EAE provides a possibility to study and interfere with mechanisms leading to protracted, relapsing and demyelinating encephalomyelitis in the rat.

In our partial characterization of autoimmune responses to CNS antigens, we focused on MBP since this autoantigen is reported to be encephalitogenic in DA rats (Mostarica-Stojković et al., 1982).

However, our observations that EAE induced with MBP or MBP peptides was rarely severe, protracted and relapsing may suggest that SPR-EAE is promoted by additional autoimmune responses towards other CNS antigens, such as the encephalitogens proteolipid protein (Yoshimura et al., 1985) and MOG (Linington et al., 1993). This notion is consistent with our data on conspicuous humoral autoreactivity to MOG in DA rats immunized with spinal cord. Since these autoantibodies react with an extracellular domain of the protein they are probably pathogenic and demyelinating; both in vitro and in vivo studies have demonstrated that anti-MOG antibodies may induce selective demyelination of central nervous system fibers (Kerlero de Rosbo et al., 1990; Vass et al., 1992) and may augment demyelination in acute and chronic T cell-mediated EAE (Linington et al., 1988, 1992). A major pathogenic role of anti-MOG antibodies in the present SPR-EAE is supported by the observed selectivity of demyelination, as it affects nerve fibers in the CNS but not in the peripheral nervous system.

At present, we and others are extending the investigation on putative encephalitogens in the DA rat to include MOG and proteolipid protein. In our present study where cell-mediated immunity to MBP (measured day 14 p.i.) and humoral immunity to MOG (measured day 32 p.i.) is evident in SPR-EAE, it is impossible to say whether both responses were elicited at the time of immunization with CNS tissue or if the MOG reactivity was induced later, as a result of ongoing destruction in the CNS. Therefore, we cannot conclude or exclude intra- or inter-molecular spreading of pathogenic immune reactions as a mechanism for SPR-EAE in DA rats, as suggested for EAE in mice (Lehman et al., 1992).

We have carefully established that SPR-EAE is reliable, by reproducing the model in DA rats bred and maintained at five different locations. It is therefore remarkable that none of the previous EAE studies in this rat strain report a protracted or relapsing encephalomyelitis (Kornblum, 1968; Gasser et al., 1973, 1975; Mostarica-Stojković et al., 1982, 1983; Vukmanović et al., 1989, 1990). Since we have used an unusual sensitization procedure it was possible that the discrepancies depend on differences in immunization protocols, e.g. the use of syngeneic instead of xenogenic spinal cord, of IFA instead of CFA, and of injection at the tail base instead of foot pad immunization. However, our systematic study on the effects of these different immunization variables on the clinical course of EAE demonstrate that any combination of them could induce SPR-EAE.

During these experiments we noted that footpad immunization causes severe and protracted inflammation in the paws which hampers the assessment of neurological deficits. It is therefore possible that earlier investigators of EAE in DA rats may not have noted SPR-EAE. Another possibility is that previous experiments were terminated too early to allow SPR-EAE detection.

An advantage with the SPR-EAE model in DA rats is that the immunization protocol we describe here is relatively lenient to the animals, since granuloma-forming mycobacteria and painful footpad inoculations are avoided. Thus, when the DA rats are immunized with foreign antigens such as ovalbumin, they do not show obvious signs of ill-being such as paw inflammation/arthritis (Lorentzen et al., 1995b), large wounds at the injection site or weight-loss. The present protocol also simplifies the context in which autoimmunity is studied because the use of syngeneic autoantigen and non-immunogenic adjuvants excludes immune reactions to foreign antigens. Furthermore, chronic/protracted forms of collagen-induced arthritis (CIA) (Larsson et al., 1990) and experimental autoimmune neuritis (Lorentzen et al., 1995a) can also be induced in the DA rat, by substituting the rat spinal cord with rat collagen type II or bovine peripheral nerve myelin, respectively. Thus, this immunization protocol can be used to study several organ-specific autoimmune diseases, which provides the possibility to compare results obtained under similar conditions in different models, e.g. production of

cytokines and antigen receptor repertoire of T cells in the disease lesions.

The DA rat is also susceptible to adjuvant arthritis, experimental autoimmune uveitis (EAU) (Lalić et al., 1983) and thyroiditis (EAT) (Rose, 1975). Thus, this strain is apparently similar to the extensively used LEW rat in being susceptible to a number of induced autoimmune diseases. However, there are notable differences between the two strains in several of these models: (i) in EAE there are temporal and quantitative differences in the cytokine mRNA expression in the CNS which relate to the neurological defects. Thus, in LEW rats, IFN-γ and interleukin-12 are associated with development of EAE while tumor growth factor β and interleukin-10 are associated with recovery from disease (Issazadeh et al., 1995a, b). In DA rats, there is a protracted expression of IFN-γ mRNA, which is temporarily lower during remissions, while mRNA for the putative disease down-regulatory cytokines tumor growth factor β and interleukin-10 is expressed at minimal levels at all stages of SPR-EAE (to be published). (ii) CIA can be induced in both DA and LEW rats with xenogenic collagen but only DA rats are susceptible when syngeneic collagen is used (Griffiths, 1988). (iii) DA rats appear more susceptible to the actions of adjuvants than are LEW rats, since DA rats develop arthritis after immunization with IFA alone (Kleinau et al., 1991), while additional adjuvants such as mycobacteria must be added to IFA (= CFA) in order to induce adjuvant arthritis in LEW rats. Taken together, these strain differences indicate a genetic difference in their susceptibility to experimental autoimmune disease. Considering that MS and EAE are most likely polygenic diseases, it is interesting to note that susceptibility to EAE in DA rats is suggested to be more dependent on non-MHC genes than in LEW rats (Gasser et al., 1975). We are currently investigating the influence of MHC and non-MHC genes on the susceptibility of DA rats to SPR-EAE.

The specificity of the immune response to MBP is similar in DA and LEW rats. Two regions of MBP, the 69–87 and 87–101 rat MBP peptides, induced the most conspicuous triggering of lymph node cells in vitro and appeared equally encephalitogenic in DA rats. The increased numbers of IFN-γ secreting cells obtained in response to these two peptides in vitro implies an in vivo expansion of reactive cells. The reasons for absence of a proliferative response to the peptides in vitro is unclear. However, discrepant results with the two ways to assess T cell activation is common, for example in MS where proliferation in response to MBP is absent while a reactivity in terms of IFN-γ is readily detectable (Olsson et al., 1990).

The 69–87 and 87–101 epitopes are also reported to be encephalitogenic in LEW rats, although in this strain the in vitro proliferative response to the 69–87 region is dominant (Offner et al., 1989). This discrepancy may be due to the fact that we used a syngeneic 69–87 peptide while previous studies have used a xenogenic epitope with the guinea pig amino acid sequence, which differ from the rat sequence in one amino acid (S instead of T in the QRTQ sequence). This residue influences binding of the peptide to LEW MHC class II molecules, $RT1B^{l}$ (Joosten et al., 1994) and proliferation of MBP reactive T cells from the LEW rat (Mannie et al., 1989). In combination with observations in both rats and mice that in vitro proliferation, of such T cells require in vitro priming with xenogenic MBP (Lehman et al., 1992) our findings may indicate that the definition of immunodominant determinants on xenogenic auto-antigens may be skewed to xenogenic epitopes when based on proliferative responses.

The fact that the same MBP epitopes are encephalitogenic in DA and LEW rats is interesting because the two strains have different MHC class II alleles, $RT1B^{a}/D^{a}$ in DA rats and $RT1B^{l}/D^{l}$ in LEW rats (Hedrich, 1990). Furthermore, we have evidence that the in vitro responses of draining lymph node cells to the 68–87 or 87–101 MBP peptide could be blocked by monoclonal antibodies towards RT1B (OX6) and RT1D (OX17) respectively (data not shown), which has also been reported in the LEW rat (Offner et al., 1986). This may indicate that the MHC class II molecules of these two strains have similar peptide binding capabilities, which may also be the case for the many other haplotypes where the 68–88 MBP epitope is immunogenic, as demonstrated in RT1 congenic LEW strains including LEW $RT1^{a}$ (AVN) (Mustafa et al., 1993).

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