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Experimental allergic encephalomyelitis induced by the peptide encoded by exon 2 of the MBP gene, a peptide implicated in remyelination

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Abstract

The discovery of T lymphocytes reactive to the peptide encoded by exon 2 of the myelin basic protein (MBP) gene in multiple sclerosis (MS) patients has drawn attention to MBP isoforms harboring that peptide as candidate autoantigens. Previously, immunological studies in MS had almost exclusively used the more abundant 18.5 kDa isoform of MBP, which does not contain the exon 2 peptide. Investigations of experimental allergic encephalomyelitis (EAE) have also focussed on the 18.5 kDa MBP isoform and its peptides. Since EAE is an animal model widely used to study MS, we examined the encephalitogenic potential of exon 2 peptide in the SJL/J mouse. Evidence for increased expression of exon 2-containing isoforms during remyelination in mouse CNS suggested that exon 2-sensitized T cells, with encephalitogenic capacity, might be important in the perpetuation of relapsing EAE (rEAE). Our experiments have demonstrated that exon 2 peptide is inherently immunogenic in SJL mice and that EAE could be induced by the adoptive transfer of exon 2-sensitized lymphocytes. Furthermore, the disease could be accentuated by the transfer of short-term exon 2-reactive lines or by a combination of adoptive transfer and antigenic challenge with exon 2 peptide. The immunodominant epitope(s) appeared to localize to the segment bordered by amino acids 59–85.

Key words: Experimental allergic encephalomyelitis; Myelin basic protein isoforms; Exon 2 peptide

1. Introduction

It is widely accepted that autoimmune processes play a critical role in the pathogenesis of multiple sclerosis (MS), a demyelinating disease of the human central nervous system (CNS). However, the specific components of myelin targeted in the initiation and/or perpetuation of disease remain to be identified. Candidate autoantigens in MS include the 18.5 kDa isoform of myelin basic protein (MBP), proteolipid protein, myelin-associated glycoprotein and myelin oligodendrocyte glycoprotein (Johnson et al., 1986; Richert et al., 1988; Ota et al., 1990; Sun et al., 1991; Martin et al., 1992b; Chou et al., 1989, 1992). Although the 18.5 kDa isoform of MBP has been extensively investigated as an autoantigen, no distinct immune reactivity to epitopes within that protein has been consistently found when MS patients were compared with healthy controls (Pette et al., 1990; Martin et al., 1990; 1992a, b). Recent data suggest that alternative isoforms of MBP should also be considered as target antigens. MBP occurs as multiple isoforms, generated by alternative splicing of exons 2, 5, or 6 from the primary MBP transcript (Kamholtz et al., 1988; Fritz et al., 1989). In the mouse, five isoforms (21.5, 18.5, 17, 17, and 14 kDa) and in the human, four isoforms (21.5, 20.2, 18.5 and 17 kDa) have been identified (Fig. 1) (Newman et al., 1987; Roth et al., 1987). The amino acid sequence encoded by the exon 2 segment of the MBP gene (exon 2 peptide), present only in human 20.2 and 21.5 kDa isoforms, was found to be recognized by $CD4^+$ T lymphocytes in MS patients as well as controls (Voskuhl et al., 1993a). Furthermore, in a multiplex MS family, the frequency of exon 2 peptide-specific T cells was higher than the frequency of T cells specific for immunodominant epitopes within the 18.5 kDa isoform (Voskuhl et al., 1993b).

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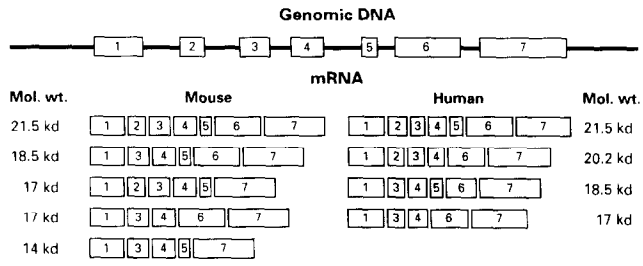

Fig. 1. Messenger RNA transcripts of the MBP isoforms in mouse and human, generated by alternative splicing of the primary transcript (modified from Fritz and McFarlin, 1989).

Since the animal model experimental autoimmune encephalomyelitis (EAE) is widely used in the study of MS (Raine et al., 1984), we investigated the encephalitogenic potential of exon 2 peptide in the SJL mouse. Exon 2-containing isoforms are present in relatively small amounts in the CNS of the adult mouse, as well as the adult human. These isoforms appear to be produced in abundance during early development and remyelination (Roth et al., 1987; Jordan et al., 1989; Kamholtz et al., 1989). Developmental studies on the accumulation of MBP isoforms in murine myelin indicate that the stoichiometric proportions of the 21.5, 18.5, 17 and 14 kDa isoforms change from 1:5:2:10, during active myelination, to 1:10:3.5:35, in adult myelin, respectively (Barbarese et al., 1978). Exon 2-containing transcripts have also been found to be up-regulated during remyelination in the murine CNS subsequent to MHV-A59 corona virus-mediated demyelination (Jordan et al., 1990). Since remyelination occurs in a cyclical manner during the course of relapsing–remitting EAE (rEAE) (Brown et al., 1982; Raine et al., 1984), this novel regulation of exon 2 expression prompted us to examine the peptide's potential as an encephalitogen. Our studies have demonstrated that exon 2 peptide-specific $CD4^+$ T cells can be isolated from animals immunized with exon 2 peptide and that these cells are encephalitogenic when adoptively transferred into naive SJL mice. Furthermore, the immunodominant epitope(s) appear to be located within the segment bordered by amino acids 59–85.

2. Materials and methods

2.1. Mice

6-week-old female SJL mice were obtained from Jackson Laboratory (Bar Harbor, ME). Mice were 8–12 weeks of age when sensitized.

2.2. Antigen

Whole myelin basic protein (gpMBP) was prepared from adult guinea pig spinal cord (Rockland Inc., Gilbertsville, PA) according to the procedure of Deibler et al. (1972). gpMBP almost exclusively consists of the 18.5 kDa isoform, which does not include the exon-2 peptide (Kerlero de Rosbo et al., 1991).

Exon 2-encoded synthetic peptides were generated by a solid phase method, HPLC purified to 98.6–99.9% purity with an aliquot undergoing confirmatory amino acid (aa) composition analysis (Synthecell/Vega Biomolecules Corporation, Columbia, MD). Two peptides were used: (i) 'exon 2 26-mer' is a 26 aa sequence corresponding to the portion of murine MBP strictly encoded by exon 2. The sequence is an follows:

VPWLKQSRSPLPSHARSRPGLCHMYK

(ii) 'Exon 2 42-mer' is a 42 aa sequence that includes a 6 aa segment and a 9 aa segment encoded by regions of the MBP gene 5′ and 3′ to exon 2, respectively. The sequence is as follows:

PKRGSGKVPWLKQSRSPLPSHARSRPGLCHMYK **DSHTRTTHY**

Concanavalin A (ConA) was obtained from Sigma (St Louis, MO) and PPD, from Connaught Laboratories (Willowdale, Ontario, Canada). 'hMBP 136–155', a synthetic peptide corresponding to aa 136–155 of the 18.5 kDa isoform of human MBP, was obtained from Synthecell (Columbia, MD).

2.3. Immunization

Quantities of exon 2 42-mer or 26-mer were dissolved in PBS and emulsified in an equal volume of complete Freund's adjuvant (CFA) (Difco Laboratories, Detroit, MI). For controls, PBS was used alone in the emulsion. Each mouse was injected subcutaneously with 30 μg of inactivated *Mycobacterium tuberculosis*, either alone or with various doses of exon 2-encoded peptide. The emulsion was distributed over four sites draining the inguinal and axillary lymph nodes. In animals used to derive MBP-reactive cells, the identical procedure was used, substituting 400 μg of gpMBP as the immunogen.

2.4. Derivation of antigen-specific cell lines

15 days after immunization, draining lymph nodes were harvested, washed and then resuspended at 4×10^6 cells/ml with either exon 2 peptide (20 μg/ml) or PPD (50 μg/ml) in tissue culture medium (TCM). Medium consisted of RPMI 1640 with 4% fetal calf serum, 1% penicillin G, 1% pyruvate, 1% non-essential amino acids, 1% glutamine, and 0.05 mM 2-mercaptoethanol. After 4 days of culture at 37°C and 5% CO_2 in 24-well cluster plates (Costar, Cambridge, MA), cells were harvested and resuspended at 1×10^{-6}/ml in TCM with $2–5 \times 10^6$ irradiated (3000 r) syngeneic spleen cells. 10 days later, viable cells were

restimulated at a concentration of 1×10^6 cells/ml with exon 2 peptide (5 μg/ml) or PPD (50 μg/ml) and irradiated APCs (5×10^6 cells/well) in 2 ml volumes for 4 days.

2.5. Proliferation assays

Lymph node cells (5×10^5 in 0.2 ml) were cultured with various concentrations of antigen or mitogen or with media alone. Cultures were maintained for 4 days in 96-well round-bottom microtiter plates (Costar, Cambridge, MA) at 37°C in 5% CO_2-air. Wells were pulsed for the final 16 h of culture with 1 μCi of [^{3}H]methylthymidine (Amersham Corp., Arlington Hts, IL). Cells were harvested on glass fiber filters and incorporated radioactivity was quantitated on a Betaplate Counter (Wallac, Gaithersburg, MD). Results were determined as arithmetic means of quadruplicate cultures; SEM was equal to or less than 15% in all assays. Stimulation index (S.I.) was calculated by dividing the mean cpm of cultures incubated with antigen or mitogen by the mean cpm of cultures incubated in media alone.

2.6. Adoptive transfer

At the end of the culture period viable cells were harvested, washed and resuspended in PBS and injected into naive SJL mice ($1.5–9 \times 10^7$ cells/mouse).

2.7. Antigenic challenge

Some mice that remained asymptomatic for 2 months following adoptive transfer were immunized with the

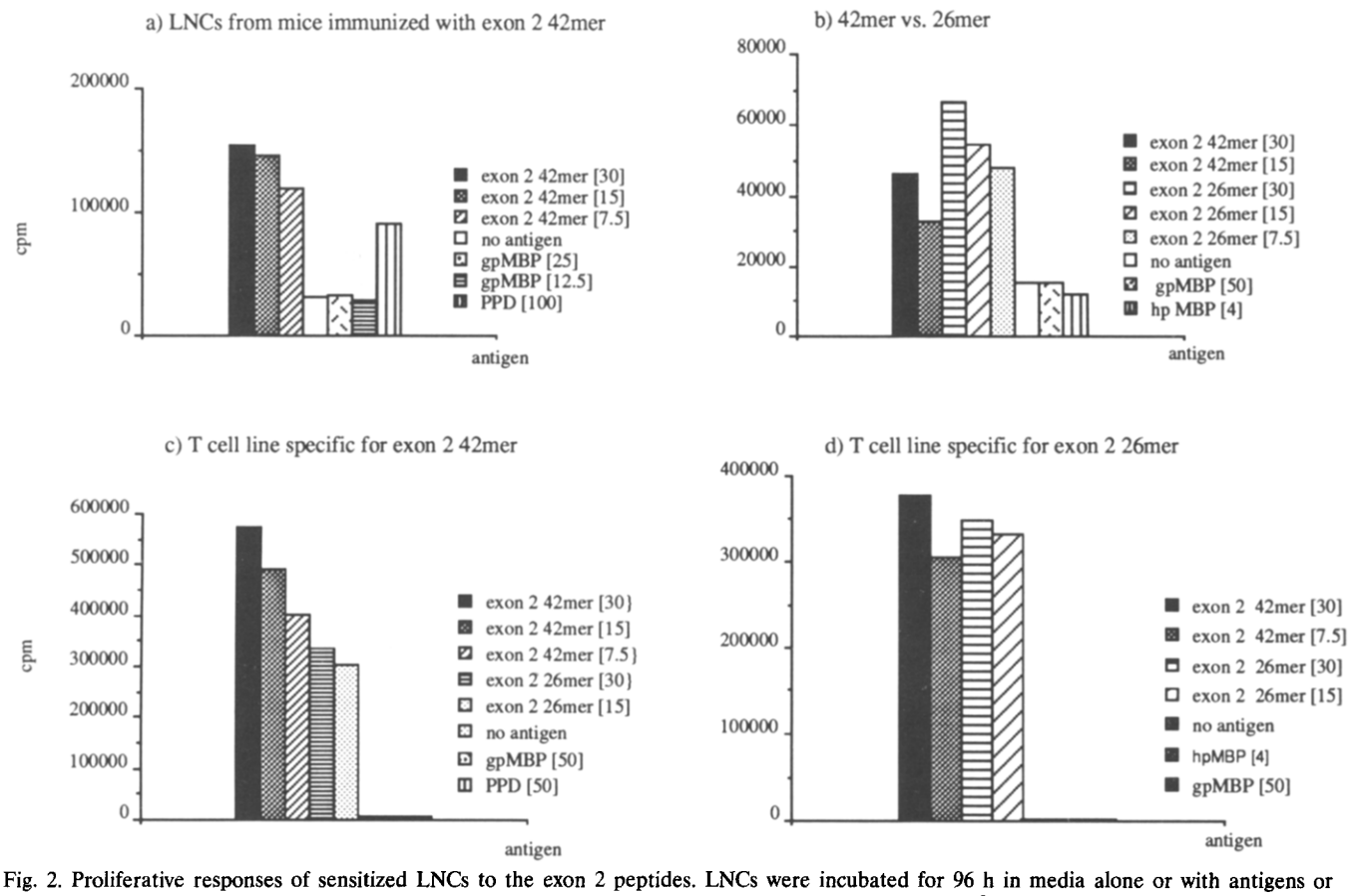

Fig. 2. Proliferative responses of sensitized LNCs to the exon 2 peptides. LNCs were incubated for 96 h in media alone or with antigens or mitogens in the concentrations indicated in the brackets, in units μg/ml. Proliferation was measured by ^{3}H-thymidine incorporation 16 h after wells were pulsed. In all assays, cultures were performed in quadruplicate with SEM less than, or equal to, 15%. **(a)** LNCs were obtained from mice immunized with exon 2 42-mer in CFA 10 days earlier. A significant response to exon 2 42-mer was found in multiple experiments, in the absence of a response to gpMBP (basically comprising the exon 2-exempt 18.5-kDa isoform). PPD was used as a positive control, as *Mycobacterium tuberculosis* is present in the immunizing emulsion. **(b)** LNCs obtained under the same conditions as **(a)** were incubated with exon 2 42-mer and exon 2 26-mer in parallel. Similar responses to the two peptides were repeatedly demonstrated. 'hpMBP', a peptide corresponding to amino acids 136–155 of the 18.5-kDa isoform of human MBP, was used as a negative control. **(c)** LNCs obtained from mice immunized with the 42-mer were stimulated twice in vitro to promote the proliferative response. The similarity in the responses to the 42-mer and the 26-mer, and the absence of reactivity to gpMBP, persisted, illustrating the specificity of these LNCs. The loss of reactivity to PPD is indicative that the protocol selected for exon 2 peptide-reactive T cells to the exclusion of other populations. **(d)** LNCs obtained from mice immunized with exon 2 26-mer were stimulated twice in vitro with the 26-mer. The pattern of reactivity is similar to that obtained in analogous experiments employing the 42-mer **(c)**.

appropriate antigen in CFA, as described in Section 2.3. The remaining mice were followed as controls.

2.8. Clinical assessment

Recipient mice were examined daily for clinical signs

Table 1
Clinical characteristics of mice injected with antigen-sensitized T cells following one cycle of in vitro stimulation

Antigen [a]	S.I. [b]	Inoculum (No. of cells $\times 10^7$)	Incidence of EAE	Day of onset [c]	Peak clinical grade [d]
Exon 2					

sensitized LNCs resembled that of mice receiving gpMBP-sensitized LNCs in parallel experiments. However, the mice in the former group invariably developed less severe disease and greater numbers of LNCs were required for disease induction (Table 1). Similar results were obtained when the 42-mer was used as the immunogen (data not shown). In an attempt to enhance the response to exon 2 peptide and, perhaps, produce more severe disease, LNCs from mice immunized with exon 2 26-mer were stimulated twice prior to transfer. For this, LNCs were incubated with exon 2 peptide for two 4-day cycles separated by a 10-day rest period, and transferred in doses of $1.5–3 \times 10^7$ cells/mouse. Phenotypic analysis demonstrated the lines to be $CD4^+$, $CD8^-$. 100% (15/15) of animals adoptively transferred with these cells became ill without further manipulation. Disease was generally severe, with the majority of mice paralyzed in the hind limbs at the peak of disease (Table 2). Animals were followed through several relapses (Fig. 5). Pathological analysis yielded findings typical of EAE, with extensive inflammatory and demyelinating involvement of the entire neuroaxis. Lesions appeared to be more purely demyelinative than most murine models of EAE (Raine et al., 1984) and axonal dystrophy and Wallerian degeneration were rarely seen (Fig. 6a–d). Twenty control animals receiving restimulated PPD-specific T cells failed to develop either clinical or histological evidence of disease (Tables 2 and 4a).

A second series of experiments to enhance the encephalogenicity of exon 2 peptide consisted of first sensitizing recipients with LNCs from 42-mer-immunized donors (cells reactive to both the 26-mer and 42-mer peptides), and, 2 months later, rechallenging those animals failing to develop EAE with a subcutaneous injection of exon 2 peptide (26-mer) in CFA. This technique, of combining adoptive transfer and antigenic challenge, was originally employed to induce EAE in 'reportedly resistant strains' (Shaw et al., 1992). All recipients in our experiments (12/12) displayed clinical signs of EAE within 8–10 days post antigenic challenge, including four mice initially injected with 3×10^7 cells, three mice injected with 6×10^7 cells and five mice injected with 9×10^7 cells (Table 3). A relapsing–remitting course ensued (Fig. 7). Histopathological examination revealed extensive inflammatory lesions along the entire neuroaxis with large plaques of ongoing demyelination apparent in the spinal cord. As before, axonal dystrophy and Wallerian degeneration were sparse (Fig. 8a–c). Macrophages laden with myelin debris were abundant in the lesions (Fig. 8b,c). A blinded observer noted the presence in these exon 2-rechallenged animals of areas of recent activity superimposed upon long-standing lesions, some of which contained remyelinated fibers (Fig. 8d), while others displayed fibrillary astrogliosis and beginning separa-

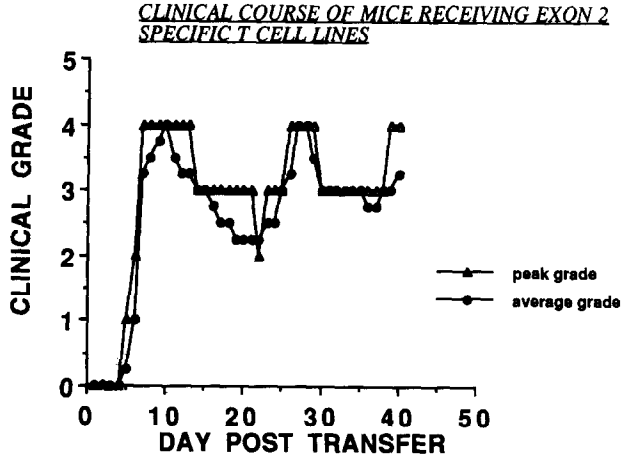

Fig. 5. Clinical course of mice injected with T cell lines specific for exon 2 peptide. LNCs obtained from mice immunized with exon 2 26-mer in CFA were stimulated twice in vitro with the 26-mer and then injected into naive recipients at $1.5–3 \times 10^7$ cells/mouse. Mice were examined daily for signs of EAE and graded on a 5 point scale of disease severity as previously described (Petinelli et al., 1981), with 4 representing hindlimb paralysis and 5 representing a moribund state. All of the recipients (15/15) developed EAE and a relapsing-remitting course followed.

fashion 6–8 days post transfer in repeated experiments and exhibited clinical features typical of EAE. 37.5% of mice (3/8) injected with 9×10^7 cells, 25% (1/4) injected with 6×10^7 cells and no mice (0/10) injected with 3×10^7 cells developed obvious neurological deficits (Table 1). Pathological examination of representative animals demonstrated meningeal inflammation at the levels of the cerebral hemispheres and spinal cord, perivascular lymphocytic cuffs distributed throughout the spinal cord, large lesions in the thoracolumbar cord comprising mononuclear inflammatory infiltrates and scattered demyelinated axons, and demyelination and inflammation of the optic nerves (Fig. 3a–d), changes consistent with EAE. Two of the animals which failed to display clinical signs after adoptive transfer with 3×10^7 exon 2 peptide-sensitized cells were killed 2–3 weeks after transfer, a time point when their symptomatic counterparts continued to exhibit deficits. Interestingly, these mice had small CNS lesions consisting of inflammatory infiltrates in the meninges of the lower spinal cord (Fig. 4). The clinical appearance of symptomatic mice receiving exon 2-

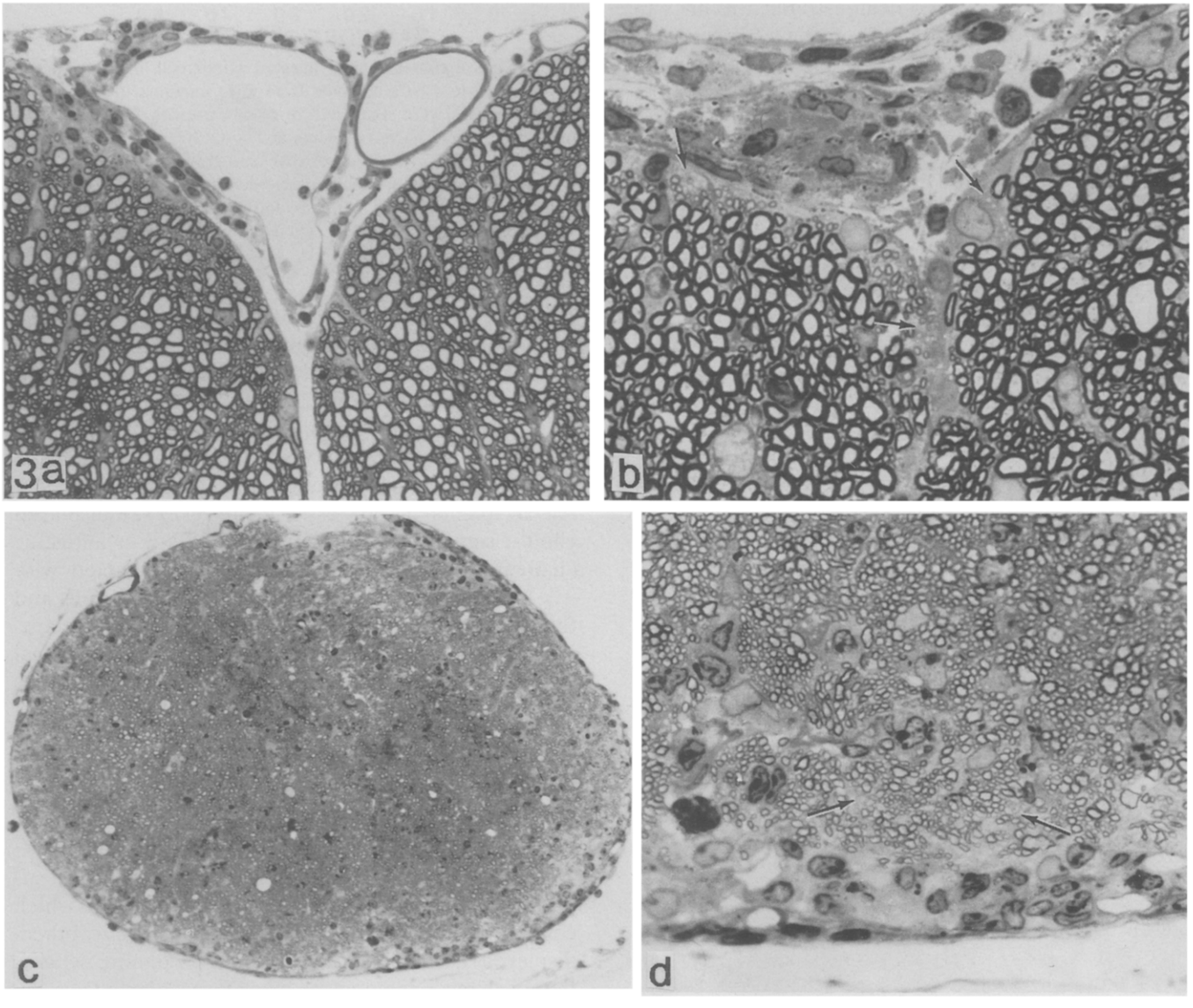

Fig. 3. (**a**) A section of lumbar spinal cord is shown from a mouse sampled 11 days after the adoptive transfer of exon 2 26-mer-specific T cells which had been stimulated once in vitro; clinical grade 1.0. Note the perivascular infiltration around the leptomeningeal vessels overlying the anterior columns and the mild edema in the subpial white matter. Toluidine blue-stained, 1-μm epoxy section. Magnification $\times$350. (**b**) A higher magnification view of the subpial region to the upper left in (**a**) shows detail of the meningeal infiltrate and groups of small demyelinated axons (arrows) in the underlying small lesion. Some astrocytic hypertrophy is also evident. Magnification $\times$875. (**c**) A section of optic nerve from an identically sensitized animal, day 9 post-transfer; clinical grade 1. Note the scattered inflammatory cells around the pial surface and the circumferential rim of diffuse demyelination. Magnification $\times$190. (**d**) Detail of an area from the margin of the optic nerve shown in (**c**) to show the cellular infiltrate, groups of small demyelinated axons (arrows) and the general hypercellularity of the parenchyma. Magnification $\times$875.

sensitized LNCs resembled that of mice receiving gpMBP-sensitized LNCs in parallel experiments. However, the mice in the former group invariably developed less severe disease and greater numbers of LNCs were required for disease induction (Table 1). Similar results were obtained when the 42-mer was used as the immunogen (data not shown). In an attempt to enhance the response to exon 2 peptide and, perhaps, produce more severe disease, LNCs from mice immunized with exon 2 26-mer were stimulated twice prior to transfer. For this, LNCs were incubated with exon 2 peptide for two 4-day cycles separated by a 10-day rest period, and transferred in doses of $1.5–3 \times 10^7$ cells/mouse. Phenotypic analysis demonstrated the lines to be $CD4^+$, $CD8^-$. 100% (15/15) of animals adoptively transferred with these cells became ill without further manipulation. Disease was generally severe, with the majority of mice paralyzed in the hind limbs at the peak of disease (Table 2). Animals were followed through several relapses (Fig. 5). Pathological analysis yielded findings typical of EAE, with extensive inflammatory and demyelinating involvement of the entire neuroaxis. Lesions appeared to be more purely demyclinative than most murine models of EAE (Raine et al., 1984) and axonal dystrophy and Wallerian degeneration were rarely seen (Fig. 6a–d). Twenty control animals receiving restimulated PPD-specific T cells failed to develop either clinical or histological evidence of disease (Tables 2 and 4a).

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Results with control animals confirmed the specificity of disease for exon 2 peptide. Mice receiving PPD-sensitized LNCs failed to develop disease follow-

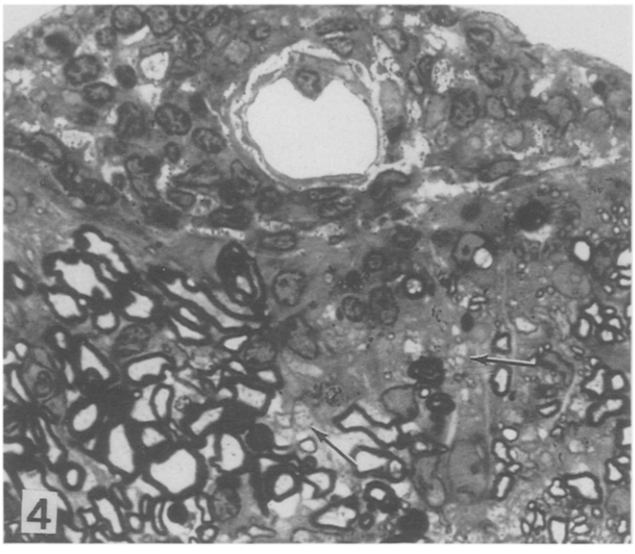

Fig. 4. A small subpial lesion is seen at the margin of the lumbar spinal cord from a mouse sensitized as in Fig. 3a which remained clinically grade 0 (asymptomatic) and was sampled on day 40 post-transfer. A perivascular cuff is shown around a vessel in the subarachnoid space overlying a small lesion in which a few demyelinated axons can be discerned. Magnification ×875.

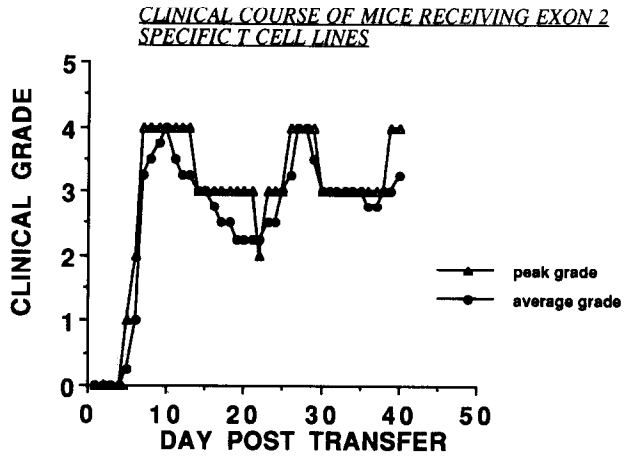

Fig. 5. Clinical course of mice injected with T cell lines specific for exon 2 peptide. LNCs obtained from mice immunized with exon 2 26-mer in CFA were stimulated twice in vitro with the 26-mer and then injected into naive recipients at $1.5–3 \times 10^7$ cells/mouse. Mice were examined daily for signs of EAE and graded on a 5 point scale of disease severity as previously described (Petinelli et al., 1981), with 4 representing hindlimb paralysis and 5 representing a moribund state. All of the recipients (15/15) developed EAE and a relapsing-remitting course followed.

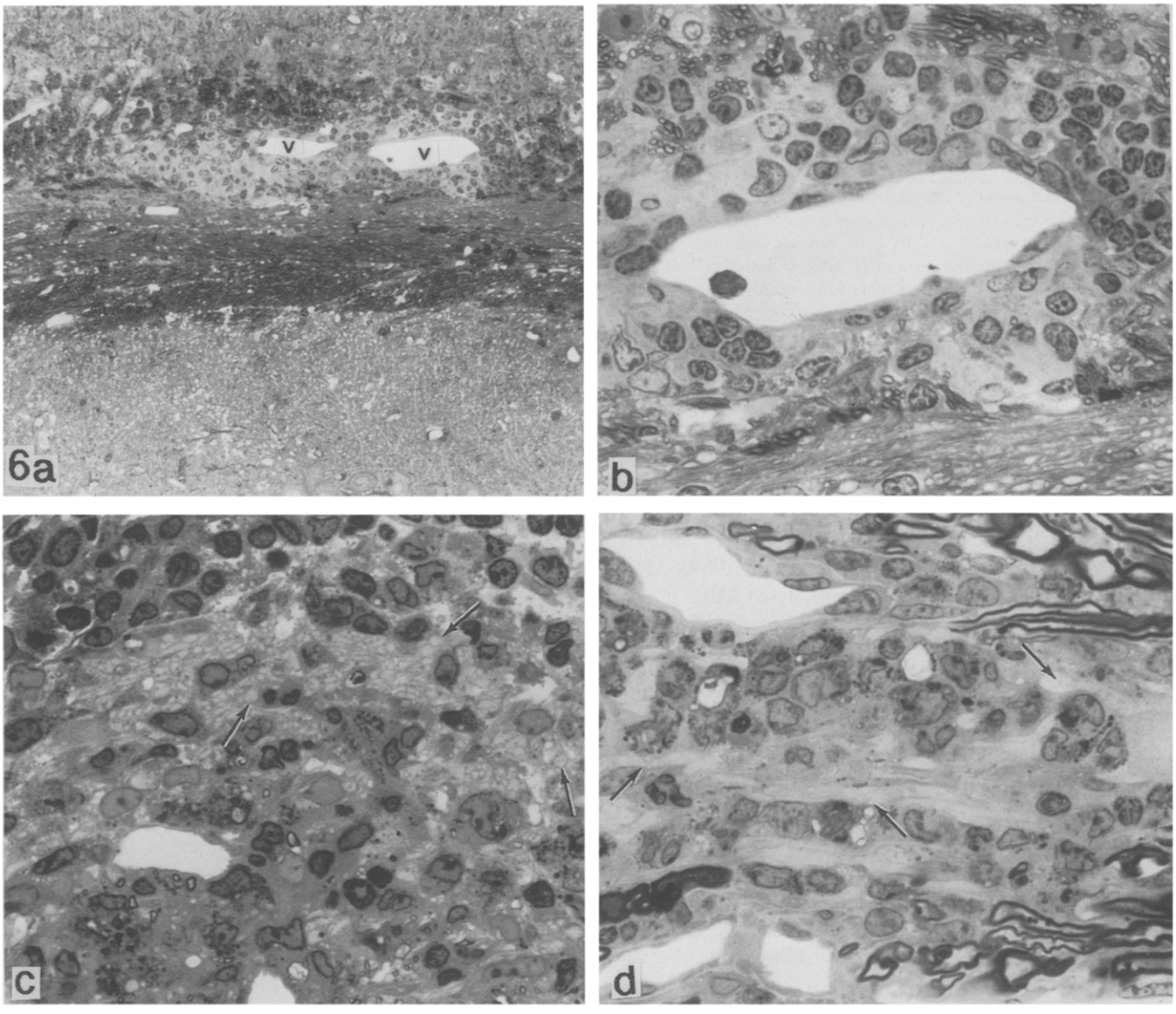

Fig. 6. (**a**) An area of corpus callosum from a mouse sampled 9 days post transfer of exon 2 26-mer-specific lymph node cells which were stimulated twice in vitro with peptide; a clinical grade 2. Note the perivascular inflammation and demyelination around the two vessels (v) and the layer of hippocampal neurons below. Magnification ×350. (**b**) Detail of the vessel to the right in (**a**) to show the nature of the cellular infiltrate, the diffuse demyelination (above), and the many longitudinally sectioned demyelinated axons (below). Magnification ×875. (**c**) Same animal as (**a**), optic nerve. Detail of a lesion at the edge of the nerve shows numerous demyelinated axons in cross section (arrows), a mononuclear infiltrate along the pial margin (above), and perivascular macrophages containing myelin debris (lower left). Magnification ×875. (**d**) Another animal from the same experiment shows a lesion in the lumbar spinal cord with demyelinated axons sectioned longitudinally (arrows). Note the many myelin-laden macrophages. Magnification ×875.

ing transfer or, subsequently, upon antigenic challenge with either exon 2 peptide in CFA or PPD alone (Table 3). Of ten mice receiving LNCs that were obtained from PPD-immunized donors and then stimulated with exon 2 peptide in vitro, one developed a weak tail upon antigenic challenge with exon 2 peptide. A meningeal infiltrate in the lower spinal cord was observed on histological analysis of this animal. None of the other control animals displayed evidence of histological disease (Table 4). In addition, animals actively immunized once with exon 2 peptide in CFA remained asymptomatic (Table 3). Controls were examined daily for over 100 days following antigenic challenge or immunization.

4. Discussion

The purpose of this study was to examine the encephalogenicity of the peptide encoded by exon 2 of the MBP gene in the SJL/J mouse. Previous studies of EAE have focussed on the abundant 18.5 kDa MBP

Table 2
Clinical characteristics of mice injected with high affinity antigen-specific T cell lines

Antigen [a]	Incidence of EAE	Day of onset [b]	Peak clinical grade [c]
Exon 2 [d]	15/15	6.6 [5–9]	3.9 [3,4]
PPD	0/20	n.a.	0

[a] LNCs from SJL/J mice immunized with antigen in CFA were stimulated twice in vitro with the same antigen and then injected into naive SJL/J mice at $1.5–3\times10^7$ cells/mouse. The quantities of antigen used for immunization and in vitro stimulation had been found to produce the strongest proliferative responses in previous experiments (data not shown).
[b] The mean number of days between adoptive transfer and the first signs of EAE is shown, with the range in brackets.
[c] Mice were graded on a five point clinical scale with 4 representing hindlimb paralysis and 5, a moribund state, as described in the text. The mean grade at the height of disease severity is shown, with the range in brackets.
[d] Exon 2 refers to exon-2-encoded peptide.

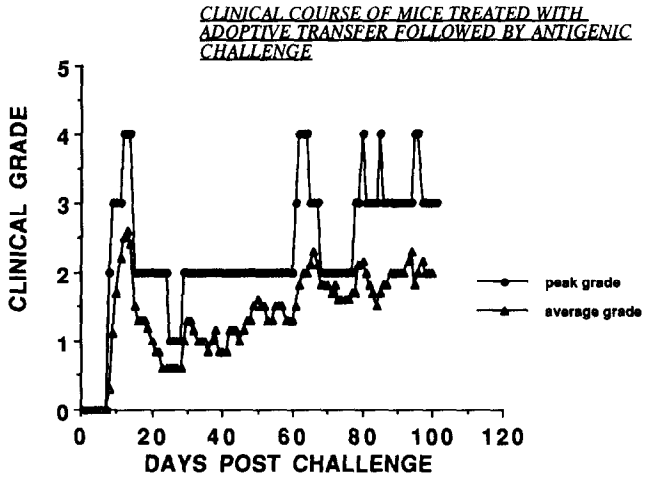

Fig. 7. Clinical course of mice treated with a combination of adoptive transfer and antigenic challenge. LNCs obtained from mice immunized with exon 2 42-mer in CFA were stimulated with the 42-mer for one cycle and injected into naive recipients at $3–9\times10^7$ cells/mouse. Animals remaining asymptomatic for 60 days post transfer were immunized with exon 2 26-mer in CFA. Mice were evaluated daily and graded for disease severity in the same manner as described in Fig. 5. All of these rechallenged animals (12/12) developed EAE at reproducible time points and a relapsing–remitting course ensued.

isoform and its peptides as encephalitogens (Petinelli et al., 1981; Fallis et al., 1989; Fritz et al., 1989). Most attempts to identify a unique reactivity to MBP in MS have also focused on the 18.5 kDa isoform (Martin et al., 1992b). Recently, however, evidence demonstrating reactivity to MBP exon 2 peptide has emerged from studies of humans (Voskuhl 1993a,b). Since expression of exon 2-containing isoforms is increased during remyelination (Jordan et al., 1990), encephalitogenic determinants within this region of the MBP molecule might contribute to disease progression in MS or in the relapsing form of EAE. The results of our experiments have demonstrated that exon 2 peptide is inherently immunogenic in SJL/J mice and that EAE could be induced by the adoptive transfer of exon 2 peptide-specific LNCs. Furthermore, the disease could be accentuated by the transfer of short-term exon 2-reactive lines or by a combination of adoptive transfer and antigenic challenge. On histological analysis the exon 2-induced lesions appeared more purely demyelinative compared with the lesions in most mouse models of EAE; axonal dystrophy and Wallerian degeneration were rarely seen. The site of the immunodominant epitope(s) was localized to the 26 aa sequence exclusively encoded by exon 2 since reactivity of sensitized LNCs, which were encephalitogenic when adoptively

Table 3
Clinical characteristics of mice treated with a combination of adoptive transfer and antigenic challenge

Antigen immunization [a]	In vitro stimulation [b]	Antigenic challenge [c]	Inoculum (no. of cells $\times 10^7$)	Incidence of EAE	Day of onset [d]	Peak clinical grade [e]
Exon 2	Exon 2	Exon 2	3	4/4	9.25(8–10)	3.0(2–4)
			6	3/3	9.00(8–10)	3.3(2–4)
			9	5/5	9.50(9–10)	3.0(2–4)
Exon 2	PPD	Exon 2	6	0/3	n.a.	n.a.
CFA	PPD	PPD	3	0/3	n.a	n.a.
			6	0/3	n.a.	n.a.
			9	0/3	n.a.	n.a.
CFA	PPD	Exon 2	6	0/5	n.a.	n.a.
			9	0/5	n.a.	n.a.
CFA	Exon 2	Exon 2	6	0/5	n.a.	n.a.
			9	0/5	n.a.	n.a.
Exon 2	–	–	–	0/10	n.a.	n.a.

[a] Naive SJL/J mice were immunized with either exon 2 peptide (exon 2) in CFA or CFA alone.
[b] LNCs were harvested 10 days following immunization and stimulated in vitro with either exon 2 or PPD for 4 days, at concentrations found to maximize proliferation (Fig. 2).
[c] Animals that remained asymptomatic for 60 days post transfer were immunized with either exon 2 in CFA or CFA alone.
[d] The mean number of days between antigenic challenge and the first signs of EAE is shown, with the range in parentheses.
[e] The mean clinical grade at the height of disease severity is shown, with the range in parentheses.

transferred, was similar using either the exon 2 26-mer or the 42-mer (containing 5′ and 3′ regions overlapping the exon 1 and exon 3 encoded sequences). No reactivity was obtained with the 18.5 kDa isoform of MBP which does not include exon 2 peptide. Hence, exon 2-encoded peptide, and more specifically the 26-mer, can be added to the list of encephalitogenic peptides in the SJL/J mouse.

Our findings have relevance to several aspects of autoimmune disease in general and EAE in particular. First, the ability to induce EAE with exon 2 peptide demonstrates that immunopathological disease could be elicited by autoantigens normally present in minute quantities in the target organ. Studies quantifying the relative amounts of the MBP isoforms in adult rodents, including mice, have shown the exon 2-containing isoforms to be at least ten times scarcer than the exon 2-exempt isoforms (Barbarese et al., 1978; Kerelo de Rosbo et al., 1991). While exon 2-containing isoforms of MBP are relatively abundant during early myelinogenesis, their rate of production falls off dramatically with maturation, assessed either by quantitative immunoblotting or by in situ hybridization analysis of myelin gene transcripts (Jordan et al., 1989; Kerlero de Rosbo et al., 1991). The low levels of antigen expressed in adult CNS tissue may explain the requirement of large numbers of exon 2-sensitized LNCs in order to adoptively transfer disease. The present findings emphasize the need to consider even minor antigens as candidate target antigens in autoimmune disease.

A second implication of our findings is that altered expression of some self antigens might heighten their importance as autoantigens under aberrant conditions. As mentioned previously, exon 2 expression has been reported to increase during remyelination. For example, using a model of virally induced demyelination, exon 2-containing transcripts have been described in the mouse spinal cord in areas of remyelination (Kristenson et al., 1986; Jordan et al., 1990) Since remyelination occurs cyclically in the course of rEAE (Brown et al., 1982; Raine et al., 1984), each cycle of remyelination could, theoretically, provide a scenario for the activation of resting exon 2-reactive cells in the CNS of diseased mice, secondary to increased presentation of exon 2 peptide. These conditions could result in exon 2-sensitized LNCs, with encephalitogenic capacity, contributing to disease progression in rEAE or, perhaps, in MS. Preliminary studies addressing the question of upregulation of exon 2-containing isoforms during the course of rEAE have been undertaken. Increased production of exon 2-containing transcripts in mice with chronic rEAE has been demonstrated by in situ hybridization performed on mouse spinal cord tissue using oligonucleotide probes specific for exon 2 mRNA (B. Segal, R. Voskuhl, L. Verma and N. Gogte, unpublished). Our present findings emphasize the need to consider other myelin constituents as candidate autoantigens which may also occur in small amounts or which may be increased during remyelination. In this regard, a charge isomer of MBP containing eight citrulline substitutions, MBP-C8, also associated with remyelination, has recently been described as being increased in white matter of MS patients (Martin, R., in preparation). T cells specific for MBP-C8 have been isolated from PBLs from MS patients as well as healthy controls (Martin et al., 1991).

The increased expression of exon 2 during remyelination also raises questions regarding the ease with which disease could be induced by immunization with exon 2 peptide in mice previously receiving LNCs sensitized with exon 2 peptide but remaining asymptomatic. Pathological examination of two asymptomatic recipients killed soon after predicted time points for disease onset revealed lesions consistent with mild encephalomyelitis, despite the fact that the animals never exhibited clinical deficits. Pathological analysis of four animals treated with a combination of adoptive transfer and antigenic challenge demonstrated lesions of varying age and remyelination at the sites of old activity which appeared to date to the time of the initial cell transfer. Thus, subclinical demyelination followed by remyelination in animals receiving exon 2-sensitized LNCs could produce upregulation in the expression of exon 2-containing isoforms and an increase in antigen presentation. This, in turn, could contribute to the heightened susceptibility of mice upon subsequent antigenic challenge.

The previous discussion emphasized the role of exon 2-specific lymphocytes in the perpetuation of autoimmune disease, rather than in its induction. In such a scenario, lymphocytic reactivity to exon 2 peptide could be considered an example of 'determinant spreading' (Lehmann et al., 1992). Determinants within exon 2 peptide might be considered 'cryptic' (Gammon et al., 1991) in that they are inaccessible to circulating LNCs under normal conditions; the peptide is scarce in the adult CNS, and the small amount of peptide present is sequestered behind the blood–brain barrier (BBB). In the course of encephalomyelitis, however, such conditions are altered. Expression of exon 2 peptide could, theoretically, be upregulated during remyelination at sites of damage; the BBB would be compromised at those same sites. Hence, the autoreactive repertoire could become skewed towards T cells specific for determinants encoded by exon 2 of the MBP gene, thereby producing a special instance of determinant spreading. In regard to other definitions of cryptic epitopes, it is unknown whether the determinants in exon 2 peptide would emerge as immunodominant following sensitization with whole exon 2-containing MBP isoforms. However, the significant proliferative response of 42-mer-sensitized LNCs to the 26-mer (Fig. 2c) suggests that

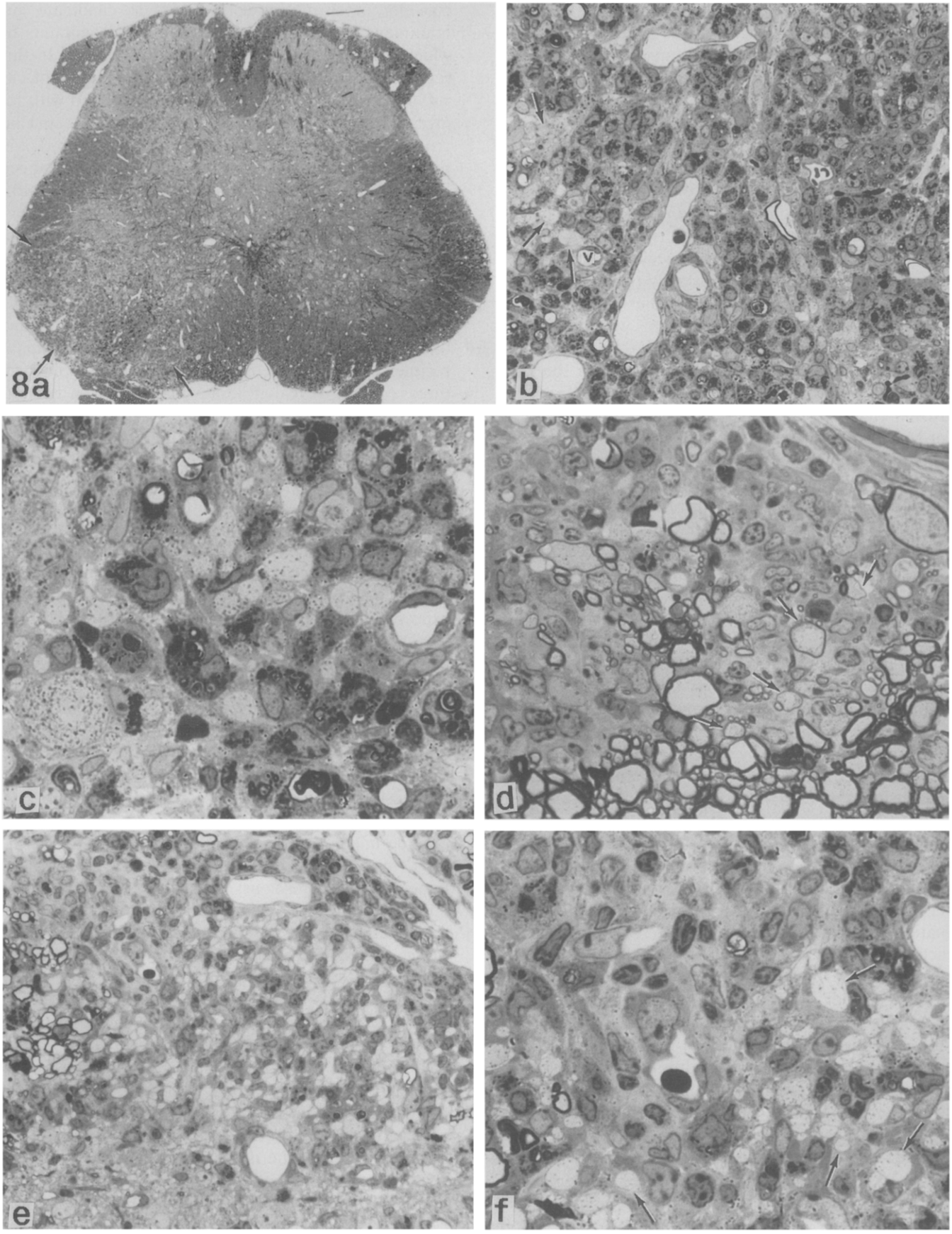
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Table 4a
A. Pathological analysis of mice injected with antigen-sensitized T cells

Mouse no.	Antigen immunization [a]	In vitro stimulation [b]	Number of cycles of stimulation [c]	Clinical grade at sacrifice [d]	Pathology [e]	
					inflammation	demyelination
1	Exon 2	Exon 2	1	2	2	1
2	Exon 2	Exon 2	1	0	1	1
3	Exon 2	Exon 2	1	0	1	0
4	Exon 2	Exon 2	2	3	3	3
5	Exon 2	Exon 2	2	2	3	1
6	Exon 2	Exon 2	2	2	2	2
7	PPD	PPD	2	0	0	0

[a–c] Naive SJL/J mice were immunized with Exon 2 peptide (exon 2) in CFA or CFA alone; LNCs were harvested ten days later and incubated with exon 2 or PPD, respectively, for either one or two 4-day cycles.
[d] Representative animals with exon 2-induced EAE were killed during exacerbations. Mice were rated for disease severity on the day of sacrifice using the five point scale described in the text. Negative control mice (ex. no. 7) were killed at time points analogous to those of symptomatic mice. Mouse no. 3 was killed 40 days following adoptive transfer, never having exhibited signs of EAE.
[e] Mice were graded according to the severity of inflammatory and demyelinating activity in CNS white matter using 5 point scales previously described (Moore et al., 1987).

Table 4b
B. Pathological analysis of mice treated with a combination of adoptive transfer and antigenic challenge

Mouse no.	Antigen immunization [a]	In vitro stimulation [b]	Antigenic challenge [c]	Clinical grade at sacrifice [d]	Pathology [e]	
					inflammation	demyelination
8	Exon 2	Exon 2	Exon 2	4	3	3
9	Exon 2	Exon 2	Exon 2	3	3	4
10	Exon 2	Exon 2	Exon 2	2	3	2
11	Exon 2	Exon 2	Exon 2	1	3	3
12	Exon 2	PPD	Exon 2	0	0	0
13	Exon 2	PPD	Exon 2	0	0	0
14	PPD	Exon 2	Exon 2	?1	2	0

[a–c] LNCs from mice immunized 10 days earlier with CFA alone or exon 2 peptide (exon 2) in CFA were stimulated in vitro for 4 days either with PPD or exon 2, and then injected into naive recipients ($3–9 \times 10^7$ cells/mouse). Mice failing to exhibit signs of EAE by 60 days post transfer were immunized with either PPD or exon 2.
[d] Mice were killed upon developing signs of EAE and rated on the 5 point scale described in the text. Asymptomatic control mice (ex. 12 and 13) were killed at time points analogous to their symptomatic counterparts. Mouse no. 14 displayed a questionably weak tail after the standard latent period between challenge and disease onset observed in animals treated exclusively with exon 2.
[e] Histological specimens of CNS tissue were rated according to the degree of inflammatory and demyelinating involvement based upon 5 point scales as outlined by Moore et al., 1987.

lymphocytes reactive to exon 2 peptide can be activated by antigen-presenting cells processing larger MBP fragments. We plan to investigate the immunogenicity of the 21.5 kDa MBP isoform in the future. Experiments are currently underway to determine the precursor frequency of exon 2 peptide-specific T cells in lymphoid tissue during various stages of rEAE with simultaneous measures of exon 2 peptide expression in the CNS.

A third implication of our findings is that the en-

Fig. 8. (**a**) A transverse section of the lumbar spinal cord of a mouse given exon 2 42-mer-specific lymph node cells and which had remained asymptomatic for 60 days post transfer, at which timepoint it was rechallenged subcutaneously with exon 2 26-mer in CFA. The animal developed signs of EAE 10 days later and was killed at clinical grade 3. Note the large demyelinated plaque (arrows) occupying most of the anterior and lateral columns to the left. Toluidine blue-stained, 1-μm epoxy section. Magnification ×35. (**b**) Detail from the lesion in (**a**) to show the widespread macrophage activity and the preserved, demyelinated axons (arrows). Magnification ×350. (**c**) Higher power from the region adjacent to the vessel (v) in (b) to show the abundance of demyelinated axons. Magnification ×875. (**d**) A spinal cord lesion from another mouse rechallenged in the same manner as (**a**) and sampled 12 days later. This lesion shows recent inflammatory activity superimposed upon long-standing pathology, including some remyelination (arrows) evidenced by thin myelin sheaths around large diameter axons. This appearance is suggestive of lesion activity in the absence of clinical signs, prior to the rechallenge. Magnification ×875. (**e**) An older, demyelinated lesion is seen in a lateral column of the lumbar spinal cord of yet another animal which was rechallenged with exon 2 26-mer 2 months after the adoptive transfer of exon 2 42-mer-specific cells, during which period the animal was asymptomatic. 10 days after rechallenge, the animal displayed EAE clinical grade 2 and was sampled for study. Note the infiltrating cells in the subarachnoid space (above), the demyelinated axons amidst glial and inflammatory cells and the lack of degenerative changes. Magnification ×350. (**f**) Higher magnification of (**e**) to show that many of the demyelinated axons are separated and invested by glial scar tissue (arrows). Also present are recent inflammatory cells and the occasional myelin-containing macrophage. This picture suggests the coexistence of acute and chronic changes within the same lesion. Magnification ×875.

cephalitogenic potential of myelin antigens is relative, as is strain susceptibility. Adoptive transfer of EAE with exon 2 peptide could not be achieved using similar numbers of LNCs to those of experiments involving the 18.5 kDa isoform. However, severe disease was readily induced by two different techniques: adoptive transfer of restimulated short-term lines and a combination of adoptive transfer and antigenic challenge. Thus, some peptides considered non-encephalitogenic might have to be reclassified as encephalitogenic following the application of different techniques for disease induction.

The murine EAE model has many parallels with the human demyelinating disease, MS, including pathological appearance and a relapsing clinical course. Lymphocytes reactive to the exon 2-encoded portion of human MBP have been derived from the PBLs of both MS patients and controls, with some multiplex family members demonstrating a higher frequency of T cells specific for exon 2 peptide than immunodominant epitopes within 18.5 kDa MBP (Voskuhl et al., 1993a,b). In addition, remyelination has been found in fresh MS plaques, potentially providing a source of newly formed exon 2-containing isoforms, analogous to the mechanism proposed in our murine model (Prineas et al., 1993; Raine et al., 1993). Though it is difficult to determine whether exon 2 peptide-specific lymphocytes participate in the pathogenesis of the human disease, the fact that they are encephalitogenic in the mouse supports the need to further investigate this possibility.

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