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Encephalitogenicity of myelin basic protein exon-2 peptide in mice

Robert B. Fritz *, Ming-Lang Zhao

Department of Microbiology, Medical College of Wisconsin, Milwaukee, WI 53226, USA

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Abstract

Immunization with a synthetic peptide with an amino acid sequence corresponding to mouse myelin basic protein exon-2 induced mild experimental allergic encephalitis (EAE) in B10.RIII mice, very mild disease in SJL/J mice and no disease in (SJL × PL)F1 hybrid mice. In contrast, adoptive transfer of an exon-2 peptide-specific T cell line from SJL mice induced severe relapsing EAE in syngeneic recipients. The T cell line was specific for exon-2 peptide and did not cross-react appreciably with an MBP preparation consisting of the 18.5 and 14-kDa isoforms. mRNA for exon-2 containing isoforms could be demonstrated in the spinal cord of SJL/J and B10.RIII mice by amplification using exon-2 and exon-4 oligonucleotide primers. On a relative basis, the level of exon-2 cDNA was lower than that of exon-1 cDNA in the same spinal cord preparations from both strains of mice.

Key words: Experimental allergic encephalomyelitis; Myelin basic protein; Myelin basic protein exon-2; Epitope; Mouse; Adoptive transfer

1. Introduction

Experimental allergic encephalomyelitis (EAE) is an autoimmune demyelinating disease of the central nervous system (CNS) induced in rodents and other species by immunization with CNS tissue or the myelin proteins, proteolipid protein (PLP) or myelin basic protein (MBP). A number of encephalitogenic epitopes have been identified from the latter proteins. Although many inbred strains of mice respond immunologically to multiple epitopes found on the proteins, generally one epitope is immunodominant for a given inbred strain of mouse. Well-characterized immunodominant MBP epitopes include residues 1–11 for H-2^u and H-2^a (Zamvil et al., 1986) mice and residues 89–101 for H-2^r (Jansson et al., 1991; Zhao et al., 1993a,b), H-2^s (Sakai et al., 1988b) and H-2^q (Cross et al., 1991) mice.

In these strains of mice EAE presents as a chronic, relapsing disease (Fritz et al., 1983; Zamvil et al., 1985). The mechanism of recovery and subsequent relapse has yet to be characterized. Alternative hypotheses include the emergence of T cells specific for new epitopes which leads to relapse or a cycling of T cell anergy. Evidence for the former includes findings of changes in T cell specificity and/or use of different MHC class II molecules during the course of the disease (Perry and Barzaga, 1987; McCarron et al., 1988; Perry et al., 1991; Lehmann et al., 1992).

Myelin basic protein is encoded by a single gene that consists of at least seven exons (Campagnoni, 1988). The gene product exists as at least six isoforms as a result of alternative RNA splicing of exons 2, 5 and 6 (deFerra et al., 1985). The isoforms vary in molecular mass from 21.5 to 14 kDa. In the adult mouse, the major protein isoforms are 14 and 18.5 kDa. These latter isoforms do not contain exon-2.

In the developing mouse brain, MBP can be detected as early as 2 days after birth (Carson et al., 1983). Maximal expression occurs concurrent with maximal myelination at 16–20 days with the 21.5- and 17-kDa isoforms present in proportionately higher amounts in the myelin membrane of young mice than in adult animals. The 17-kDa isoform is composed of a mixture of two molecular forms, one of which has exons 1, 2, 3, 4, 5 and 7 and the second exons 1, 3, 4, 6

* Corresponding author. Phone (414) 257 8414, Fax (414) 257 8427

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and 7. The 21.5-kDa isoform is composed of exons 1, 2, 3, 4, 5, 6 and 7. Thus, isoforms that express exon-2 are more predominant during myelination.

A question of interest is whether exon-2-containing isoforms are expressed at higher levels during remyelination in the CNS. Jordan et al. (1990) examined this issue in mice recovering from infection and demyelination by the A59 strain of mouse coronavirus. Using synthetic oligonucleotide probes specific for MBP exon-1 and 2, they reported that expression of exon-2 mRNA was increased during remyelination and that expression was lesion-specific.

Thus, the possibility exists that a similar alteration in isoform expression is found during remyelination following autoimmune attack in the CNS. If so, T cells could be sensitized to epitopes associated with MBP exon-2 and that this could contribute to relapsing disease. This has been hypothesized to occur in the Multiple Sclerosis (Voskuhl et al., 1993). However, little is known about the immunogenicity of the MBP exon-2 region.

To address the issue of exon-2 peptide immunogenicity, SJL, (SJL × PL)F1 and B10.RIII mice were immunized with a synthetic peptide corresponding to mouse MBP exon-2. Additionally, an exon-2-specific T cell line was generated from immune SJL/J mice and used in adoptive transfer experiments. The results of these experiments are described below.

2. Materials and methods

2.1. Mice

SJL/J and B10.RIII(71NS)/SnJ mice were purchased from the Jackson Laboratory, Bar Harbor, ME. (SJL/J × PL/J)F1 hybrid mice were bred in the Animal Resource Center of the Medical College of Wisconsin. Mice 6–10 weeks of age were used for these experiments. Immunizations were with 200 μg of MBP or peptide emulsified in incomplete Freund's adjuvant supplemented with 50 μg/ml *Mycobacterium tuberculosis*, H37Ra. Immediately and 48 h after injection, 400 ng of *Bordetella pertussis* toxin (Sigma Chemical Company) was injected i.p. The mice were examined daily and scored for clinical signs on a scale from 1 (tail weakness or mild limb weakness) to 4 (severe paraparesis). Mice beyond grade 4 were killed to prevent undue suffering.

2.2. Cell culture

Inguinal and axillary lymph nodes were removed from peptide-immune SJL/J mice, disrupted in a glass homogenizer, washed, erythrocytes were lysed with ammonium chloride and the remaining cells placed in culture at 3×10^6 per ml with 10 μg/ml peptide. Incubation was carried out at 37°C for 4 days. The growth medium was RPMI 1640 supplemented with 10% fetal bovine serum (Gibco/BRL), 10 mM HEPES buffer, 0.5 mM sodium pyruvate and 5×10^{-5} 2-mercaptoethanol. At the end of the incubation period the peptide-specific T blasts were separated on Ficoll/Hypaque (density 1.077 g/ml), washed, enumerated and an aliquot of the separated cells injected into each irradiated (500R) recipient. A second aliquot was saved for flow cytometry (FCM). The remaining T blasts were cultured at 1×10^5 per ml with 1×10^6 irradiated (3000 R) syngeneic spleen cells per ml for 10 days. At the end of this time live cells were placed in culture at 1×10^5/ml with irradiated syngeneic spleen cells at 2.5×10^6/ml and 10 μg/ml peptide. After 4 days the T blasts were separated on Ficoll-Hypaque, an aliquot removed for FCM, a second aliquot used to test for encephalitogenicity and the remaining cells returned to the rest cycle for 10 days. At each stimulation microcultures were established to measure antigen-specific proliferation. Adoptive transfer of EAE was done by injection of 2×10^7 density gradient-purified T blasts intravenously into sublethally irradiated (5 Gy) syngeneic recipients.

2.3. Myelin basic protein

Myelin basic protein was purified from rat brains (Pel-Freeze, Rogers, AK) (Chou et al., 1977). SDS-polyacrylamide gel electrophoresis showed that the preparations consisted of the 14- and 18.5-kDa isoforms. Synthetic exon-2 peptide (deFerra et al., 1985) was prepared in the core facilities of the Medical College of Wisconsin (Fig. 1A). Purity was assessed by HPLC analysis and amino acid composition.

2.4. Cell surface phenotype

The surface phenotype of exon-2-stimulated blast cells was determined with commercially obtained antibodies including FITC-labeled monoclonal anti-CD3,

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V-P-W-L-K-Q-S-R-S-P-L-P-S-H-A-R-S-R-P-G-L-C-H-M-Y-K

Ⓑ

SENSE	5'-CAT-CCT-TGA-CTC-CAT-CGG-GCG-C-3'	EXON 1
SENSE	5'-TGG-CTA-AAG-CAG-AGC-CGG-AGC-3'	EXON 2
ANTISENSE	5'-TTG-GGA-TGG-AGG-TGG-TGT-TCG-3'	EXON 4

Fig. 1. (A) Amino acid sequence of mouse exon-2 peptide used in these experiments (deFerra et al., 1985). The single-letter amino acid code is shown. (B) Oligonucleotides used as primers for amplification of spinal cord cDNA.

CD4 and CD8 (Pharmingen, Inc.). Five hundred thousand blast cells were incubated with each antibody preparation for 30 min, washed and assayed in a Coulter Profile instrument.

2.5. Polymerase chain reaction

Spinal cords were removed from normal mice and total RNA extracted by the method of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). The RNA concentration was determined by optical density readings at 260 nm. 1 μg of total RNA was incubated with 10 μl 5 × buffer, 5 μl poly-T (20 μM) 2.5 μl dNTPs (10 mM) and water to a final volume of 47 μl. The mixture was heated at 68°C for 2 min, cooled in ice and 0.5 μl of RNAguard (Pharmacia), 5 μl of dithiothreitol (0.1 M) and 2.5 μl Moloney murine leukemia virus (MMLV) reverse transcriptase added. The mixture was then incubated at 42°C for 45 min.

Amplification of cDNA was done as follows. One μl of cDNA was incubated with 2.5 μl 10 × buffer, 0.5 μl dNTPs (10 mM), 1 μl 50 mM $MgCl_2$, and water to a volume of 24 μl. The mixture was heated to 98°C for 2 min, cooled in ice and 1 μl (1 unit) Taq polymerase was added. The amplification program was 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. At the end of the program there was a final extension at 72°C for 7 min. To ensure that approximately equal amounts of cDNA were used for starting material, several dilutions of each cDNA were run for a varying number of cycles with actin and HPRT primers so that the amplified products could be removed during the logarithmic phase of amplification.

MBP exon-1- and exon-2-specific primers were used with an exon-4-specific primer for amplification of the corresponding MBP cDNA. Amplification with exon-1 and exon-4 primers would yield products of 199 and 275 bp. Exon-2 and exon-4 primers would give a only a 201-bp amplification product. Estimation of the ratio of exon-1-specific products to that of exon-2-specific product was done as described above to ensure that the estimate was done in the logarithmic phase of amplification. Oligonucleotide primer sequences are shown in Fig. 1B.

3. Results

B10.RIII mice, a strain that is very susceptible to the induction of MBP-induced EAE, were weakly susceptible to EAE induced by immunization with exon-2 peptide (Table 1). As was previously noted, male mice of this strain are significantly more susceptible than females. Clinical disease was quite mild in these animals. SJL/J and (SJL × PL)F1 hybrid mice were quite resistant to induction of EAE when immunized with the peptide. In a second experiment 3/9 SJL/J mice exhibited extremely mild signs following immunization with the peptide.

Since adoptive transfer experiments give more consistent data in murine EAE than direct immunizations, a T cell line was established from lymph nodes of exon-2-immune SJL mice. A primary line was prepared and tested for encephalitogenicity by adoptive transfer of 2×10^7 Ficoll-Hypaque-separated blasts into syngeneic recipients. Three of 3 recipients exhibited signs of moderate to severe disease (Fig. 2) with an onset at day 7. This onset is typical of what is seen with MBP-specific T cell lines. One mouse was killed during acute disease due to severe symptoms. The two remaining mice recovered from acute disease and then relapsed at day 24–26. Both had reached grade 3 by day 28 approximately 2 weeks after they had begun to recover from acute disease. Again, this is typical of the course of disease induced by MBP-specific T cells (Fritz et al., 1983).

Table 1
Induction of EAE in mice immunized with MBP exon-2 peptide or MBP

Strain	Ag	Dose [a]	EAE [b]	Onset [c]	Index [d]
B10.RIII	Exon-2	200	4/10 (m) [e]	8.0	1.5
		200	0/5 (f)	–	–
		100	2/4 (m)	11.0	1.0
		100	0/4 (f)	–	–
	MBP	200	9/9 (m)	8.8	3.4
SJL/J	Exon-2	200	1/10 (f)	10.0	1.0
	MBP	200	3/5 (f)	12.0	1.3
(SJL × PL) F1	Exon-2	200	0/4 (m)	–	–
		200	0/4 (f)	–	–
	MBP	200	2/3 (f)	11.0	2.0

[a] μg antigen per animal.
[b] EAE/number injected.
[c] Day of first clinical signs.
[d] Disease index.
[e] (m), male; (f), female.

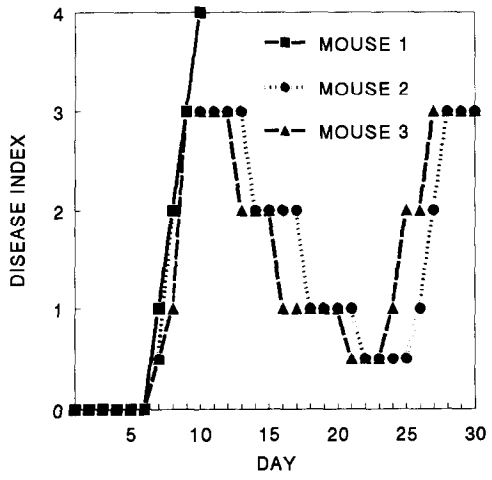

Fig. 2. Clinical disease in mice following adoptive transfer of 2×10^7 MBP exon-2-specific T blasts. Mouse 1 was killed on day 10 due to severe symptoms.

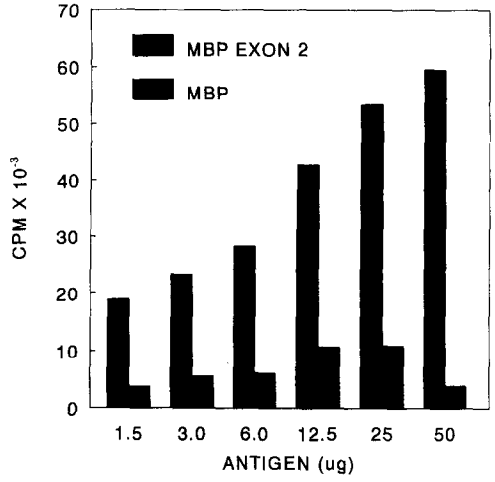

Fig. 3. Proliferative response of the MBP exon-2-specific T cell line when tested against MBP exon-2 or MBP. Data are corrected for radioactivity incorporated in the absence of antigen (8797 cpm).

Analysis of the primary exon-2-specific T blasts by microcytofluorimetry showed that the T cell population was 87% $CD3^+$, 59% $CD4^+$ and 31% $CD8^+$. These data are comparable to those from a primary MBP-specific T cell line (Zhao et al., 1993a,b).

The specificity of the line was examined by the in vitro proliferation assay. T cells from the exon-2-specific T cell line were cultured with varying concentrations of either exon-2 peptide or MBP. The results are shown in Fig. 3. The line was highly specific for exon-2 with only minimal reactivity when cultured with MBP. As a control an encephalitogenic MBP-specific T cell line was tested in the same manner. This line did not react with exon-2 peptide (not shown).

The finding that exon-2-specific T cells caused clinical disease in recipients implied that expression of exon-2 in the CNS was sufficient to provide an adequate stimulus for the transferred cells. To examine this issue, RT-PCR was done on cDNA prepared from total spinal cord RNA. Primers used were exon-1- and exon-2-specific with the reverse primer being exon-4-specific.

In this experiment, amplification was carried out for 30 cycles on cDNA from the spinal cord of a B10.RIII mouse or a SJL/J mouse. The cDNA was diluted 1:50, 1:250 and 1:1250 prior to addition to the reaction mixture. Fig. 4 shows the results of agarose gel analysis of the amplified products. Both primer sets yielded an amplified product of approximately 200 bp. This is the predicted size based on amplification of cDNA containing exon-1, 3 and 4 when incubated with exon-1 and 4 primers. Exon-2 containing isoforms would yield an amplification product of approximately the same size when incubated with exon-2 and 4 primers. Comparison of the results with the cDNA dilutions shows that there was significantly less mes-

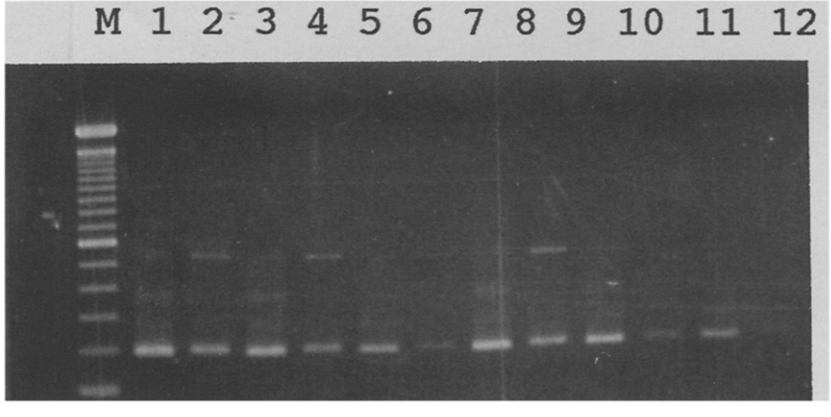

Fig. 4. Results of amplification of cDNA from B10.RIII (lanes 1–6) or SJL (lanes 7–12) spinal cord. Lanes 1, 3 and 5 were a 1:50, 1:250 and a 1:1250 dilution of cDNA, respectively, amplified with exon-1 + exon-4 primers. Lanes 2, 4 and 6 were a 1:50, 1:250 and a 1:1250 dilution of cDNA amplified with exon-2 + exon-4 primers. Lanes 7, 9 and 11 were a 1:50, 1:250 and a 1:1250 dilution of cDNA, respectively, amplified with exon-1 + exon-4 primers. Lanes 8, 10 and 12 were a 1:50, 1:250 and a 1:1250 dilution of cDNA amplified with exon-2 + exon-4 primers. All lanes also contained primers for mouse β-actin (540 bp). Markers were 100-bp ladder.

sage for exon-2-containing isoforms as would be expected in an adult mouse spinal cord.

An additional band of approximately 275 bp would be predicted to be present in the lanes where exon-1 primer had been used for amplification since any exon-2 containing cDNA should also be amplified by an exon-1 primer. A band of this size was present, but was extremely faint. This may be due to competition for reagents by the different cDNAs present which results in poor amplification of the cDNA that contains both exon-1 and exon-2. This interpretation is supported by the fact that coamplification with β-actin primers, which give a 540-bp product, results in much less signal in the tubes with exon-1 primers than in the tubes that contain exon-2 primers (Fig. 4). Both the 199-bp band and the fainter 275-bp band hybridized with a ^{32}P-labeled exon-3-specific probe (not shown). Thus, exon-2 message is present in the spinal cord of the adult mice. Presumably this is translated and expressed in the myelin membrane.

4. Discussion

Not very long ago it was thought that the T cell response to MBP was highly restricted and directed predominantly against a single immunodominant epitope (Zamvil et al., 1986; Acha-Orbea et al., 1988). Favorable interactions between a particular MBP peptide and class II MHC proteins expressed by antigen-presenting cells (APC) of the inbred mouse strain is thought to result in immunodominance. This paradigm was based on results obtained with a single MHC haplotype, H-2^u. Increasingly, it has become evident that the paradigm must be expanded to include the role of other epitopes associated with MBP in addition to the immunodominant epitope. Two recent publications serve to emphasize this point. Lehmann and colleagues (1992) reported that during the course of EAE in H-2^u mice immunized with the immunodominant peptide Ac1–11, T cells with specificities directed against other subdominant epitopes appeared. Bell et al. (1993) found that the spinal cord of (PL × SJL)F1 mice immunized with the immunodominant peptide Ac1–11 contained a very diverse collection of TCR Vβ chains. No predominant expression of any particular Vβ chain was found even though Vβ8.2 was over-represented by Ac1–11-specific T cell clones derived from this strain combination in earlier studies (Acha-Orbea et al., 1988; Zamvil et al., 1988). The difference observed between TCR expression in the spinal cord following active immunization and dominant expression of Vβ8.2 in in vitro cultures may be related to selective pressures of in vitro cultivation. We have shown that several Vβ families, including Vβ8.1 and 8.2, become significantly less heterogeneous during the course of long-term culture (Fritz et al., 1993).

The present work serves to reinforce the concept of epitope diversity in the encephalitogenic response to MBP. Previously, it has been found that there are a number of MBP epitopes that are capable of induction of EAE in the SJL strain (Pettinelli et al., 1982; Kono et al., 1988; Sakai et al., 1988a; Fritz et al., 1990; Su and Sriram, 1992). To this list may be added an epitope or epitopes associated with MBP exon-2.

Some MBP peptides are capable of induction of EAE in immunized animals following direct immunization. Others such as MBP peptide 17–27 are not effective when given directly although T cells derived from mice immunized with the peptide are capable of transferring passive disease to syngeneic recipients (Fritz et al., 1990). MBP exon-2 peptide is very weakly encephalitogenic in SJL mice; however, a T cell line derived from exon-2 peptide-sensitized SJL mice transfers disease efficiently. This may be due to a low precursor frequency for exon-2 peptide in non-immune mice. Expansion of peptide-specific T cells during in vitro culture provides sufficient T cells to allow adoptive transfer of the disease.

This raises the issue as to whether any epitope of MBP can be encephalitogenic under the appropriate conditions. Although a peptide may not appear to be encephalitogenic upon immunization, expansion of a small number of antigen-specific T cells may result in sufficient cells to cause disease. The means of expansion of the T cell population could either be specific or non-specific dependent upon the circumstances.

The recent demonstration that there are exon-2-reactive T cells in the circulation of patients with multiple sclerosis is of relevance to the present study (Voskuhl et al., 1993). It was hypothesized that exon-2-containing isoforms might be expressed during remyelination in these patients and that these isoforms might provide a source of new epitopes for T cell sensitization. The finding that exon-2 peptide is encephalitogenic in mice lends support to this hypothesis.

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