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A novel candidate autoantigen in a multiplex family with multiple sclerosis: prevalence of T-lymphocytes specific for an MBP epitope unique to myelination

Rhonda R. Voskuhl, Dale E. McFarlin, Laura R. Tranquill, Gladys Deibler, Roger Stone, Heidi Maloni and Henry F. McFarland

Neuroimmunology Branch, National Institutes of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD, USA

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Summary

Although the major isoform of myelin basic protein (MBP) in the healthy adult CNS is the 18.5-kDa protein, other isoforms containing exon 2 encoded protein (21.5 kDa and 20.2 kDa) exist and are expressed primarily during myelin formation. Since remyelination is a prominent feature in MS lesions, we examined the frequencies of T cell lines (TCLs) specific for epitopes within exon 2 encoded MBP (X2MBP), and also within 18.5-kDa MBP, in members of a multiplex family with MS. TCLs specific for X2MBP were as prevalent as TCLs specific for immunodominant epitopes within 18.5-kDa MBP. In addition, while frequencies of TCLs specific for 18.5-kDa MBP were no different between the affected and unaffected, the frequency of X2MBP-specific TCLs correlated with disease.

Introduction

Multiple sclerosis (MS) is widely accepted as an autoimmune disease, and although myelin basic protein (MBP) is a candidate autoantigen, investigation of T cell responses to the 18.5-kDa isoform of MBP has not identified an epitope which is consistently recognized with higher frequency in all MS patients compared to controls (Chou et al., 1989; Richert et al., 1989; Martin et al., 1990, 1992; Ota et al., 1990; Pette et al., 1990). In the study of a more genetically homogeneous population, a multiplex family with MS, an epitope within the 18.5-kDa MBP isoform was also not recognized by T cell lines (TCLs) more frequently in affected siblings compared to unaffected siblings (Voskuhl et al., 1993a). Previous characterization of

immune responses to MBP have focused on the 18.5-kDa isoform, although four isoforms of MBP have been described in humans. The molecular weights are 21.5 kDa, 20.2 kDa, 18.5 kDa, and 17.3 kDa (Roth et al., 1987) with the healthy adult human CNS composed primarily of the 18.5-kDa isoform. The 18.5-kDa isoform is encoded by exons 1 and 3 through 7, with a splicing out of exon 2 (Fig. 1). We have previously described CD4+, HLA class II-restricted T lymphocytes specific for a protein encoded by exon 2 of MBP (Voskuhl et al., 1993b). Exon 2 containing isoforms, 21.5 kDa and 20.2 kDa, are thought to be expressed early in fetal CNS development in both the human (Roth et al., 1987; Kamholtz et al., 1988) and mouse (Newman et al., 1987; Jordan et al., 1989). Exon 2 containing transcripts have also been found in relatively increased amounts during remyelination subsequent to MHV-A59 coronavirus mediated demyelination in C57BL/6N mice (Kristensson et al., 1986; Jordan et al., 1990). Since MBP isoforms expressed during remyelination may parallel those expressed during developmental myelination, we postulated that exon 2

Correspondence to: R.R. Voskuhl, Neuroimmunology Branch, National Institutes of Neurological Disorders and Stroke, National Institutes of Health, Building 10, Room 5B-16, Bethesda, MD 20892, USA.

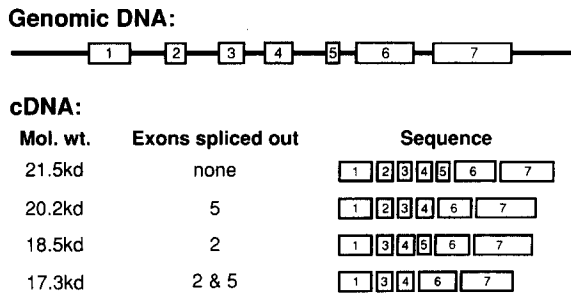


Fig. 1. Germline DNA for MBP gene which yields four cDNA isoforms through alternative splicing (modified from Fritz and McFarlin, 1989).

encoded protein might be expressed in the adult human CNS during remyelination. Since remyelination occurs at the edge of MS plaques (Prineas and Connell, 1979; Raine and Scheinberg, 1988), T lymphocyte reactivity specific for X2MBP could contribute to the pathogenesis of chronic progressive MS. This report compares the frequencies of X2MBP-specific T lymphocytes derived from the peripheral blood of MS patients and controls. In experimental allergic encephalomyelitis (EAE), an animal model resembling MS, each inbred animal's major histocompatibility complex (MHC) genotype confers susceptibility to disease as well as determining which epitope induces a T cell response (Fritz and McFarlin, 1989). Thus, a potential difficulty in identifying disease specific differences in T cell responses to myelin antigens lies in the genetic heterogeneity of human population studies (Voskuhl et al., 1993a). In order to study a more genetically homogeneous human population, we focused our study on members of a multiplex family with MS. Three family members were studied including one with more advanced secondary chronic progressive MS, one with mild relapsing remitting MS, and one unaffected. An unrelated healthy control that was age, sex, and HLA matched with the advanced secondary CP MS patient was also studied.

Materials and Methods

Patients

Three individuals from a multiplex family with MS were studied. A female (MS-CP), age 39, had advanced secondary CP MS with an expanded disability status scale (EDSS) of 6.5. Her mother (MS-RR), age 59, had mild RR disease with an EDSS of 3.0. Cerebral MRIs reflected clinical disease burden (Fig. 2A,B). The patients had not been on immunosuppressive medication within 2 years of being studied. The third family member (NL-sib) was the brother of MS-CP and the son of MS-RR who was 30 years of age, and healthy both clinically and paraclinically (Fig. 2C). An additional

healthy control (NL-match) was unrelated but matched for age, sex, and HLA class II with MS-CP (see HLA and clinical profiles, Table 1). The research was reviewed and approved by the Institute Clinical Research Subpanel and informed consent was obtained from all patients.

Cells

Peripheral blood lymphocytes (PBL) were obtained by leukapheresis and isolated by sodium-metrizoate density gradient. Cells were HLA typed by a standard NIH lymphocytotoxicity assay for HLA-A, -B, -DR, and -DQ at the Department of Transfusion Medicine, National Institutes of Health, Bethesda, MD.

Antigens

Human exon 2 encoded protein, X2MBP, was synthesized by solid phase method, HPLC purified to 99.2% purity, with an aliquot undergoing confirmatory amino acid composition analysis (Synthecell Laboratories, Rockville, MD). In addition to the 26 amino acids encoded by exon 2, the exon 2 protein included sequences of the 5' and 3' regions to provide seven amino acid overlapping regions with exon 1 and exon 3 encoded protein respectively (Fig. 3). Thus, an epitope bridging a terminal sequence of exon 2 encoded protein and exon 1 or 3 encoded protein would be detectable. Human 18.5-kDa MBP was prepared as described previously (Deibler et al., 1972). Human MBP fragments 1-98 and 99-172 were generated by thrombin cleavage as described (Law et al., 1984) and peptide 87-106 was synthesized by solid phase and HPLC purified to greater than 90% purity (Synthecell Laboratories, Rockville, MD).

Establishment of X2MBP-specific and 18.5-kDa MBP-specific T cell lines and proliferation assays

TCLs were generated from each individual by seeding 2×10^5 PBLs per 200- μ l well with X2MBP (10 μ g/ml) in complete culture media, a total of 1832 cultures from all individuals. A dose response proliferative assay confirmed 10 μ g/ml as the optimal X2MBP concentration (not shown). Cultures using 18.5-kDa MBP (10 μ g/ml) as antigen were generated in parallel under identical conditions for each individual with a total of 1832 cultures from all individuals. Each of the cultures was then screened for X2MBP- and 18.5-kDa MBP-specific proliferation (Martin et al., 1992; Voskuhl et al., 1993b). Cell cultures were considered positive for X2MBP-specific proliferation, or analogous 18.5-kDa MBP-specific proliferation, if they had stimulation indices (SI) of greater than two times control (media alone). Lines positive in the initial screening proliferation assay were restimulated with appropriate antigen, IL-2, and autologous irradiated PBLs before being retested for proliferation in triplicate. Lines were

thereby confirmed in triplicate assay as X2MBP-specific if they demonstrated an SI > 2 as compared to 18.5-kDa MBP and media controls. Lines were considered posi-

tive for 18.5-kDa MBP-specific proliferation if they had an SI > 2 as compared to X2MBP and media controls. 18.5-kDa MBP-specific TCLs were then retested in

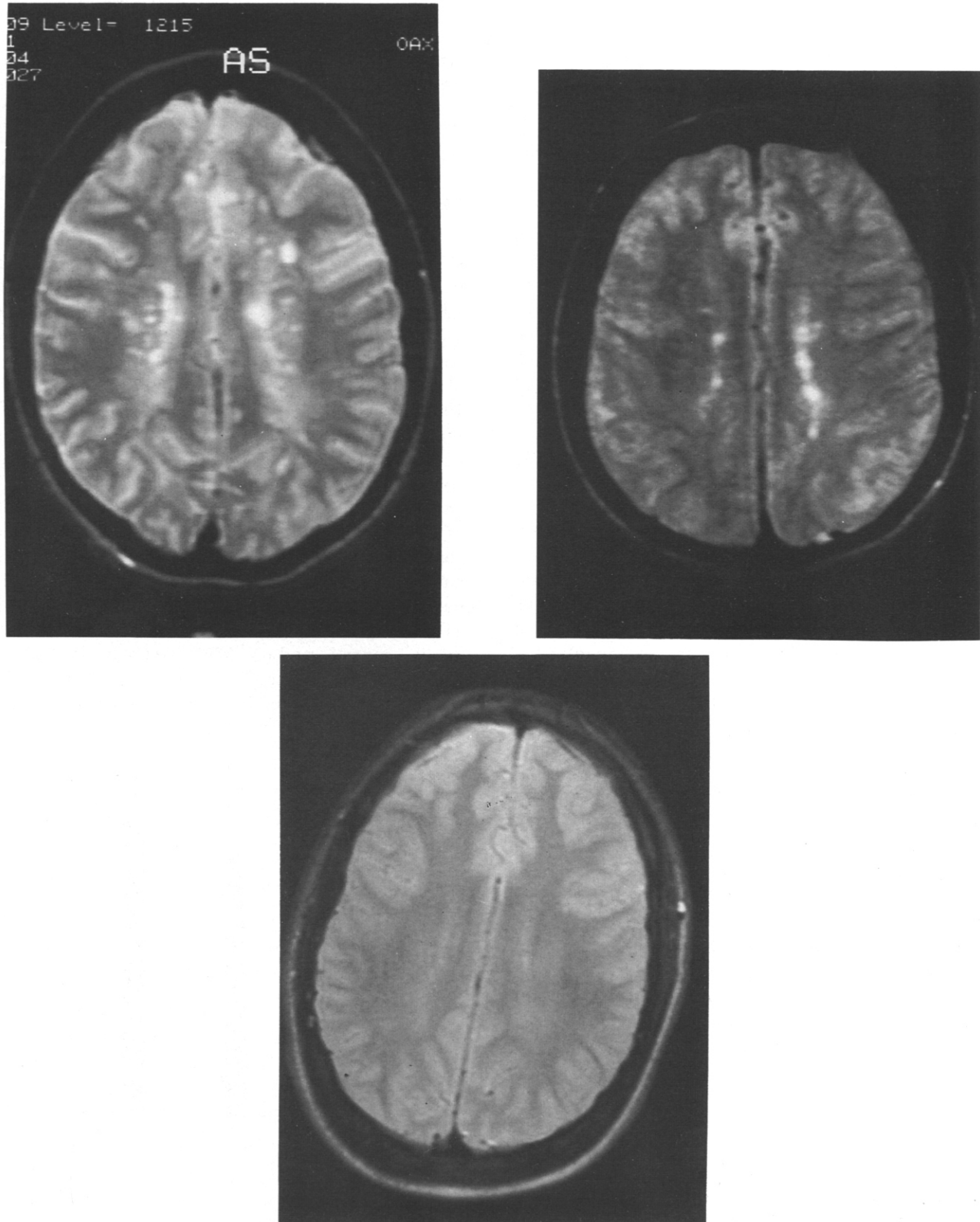


Fig. 2. Cerebral MRIs (T2 weighted image) of each member of the multiplex family. (A) MS-CP. (B) MS-RR. (C) NL-sib. Gadolinium enhancement present only in MS-CP (not shown).

TABLE 1

Patients

	Age	Sex	EDSS	HLA-A	HLA-B	HLA-DR	HLA-DQ
MS-CP	39	F	6.5	3, 31	27, 44	2, 4	1, 3
MS-RR	59	F	3.0	11, 31	27, 35	2, 1	1, 1
NL-sib	30	M		2, 31	27, 7	2, 1	1, 1
NL-match	39	F		2, 26	39, 62	2, 4	1, 3

Age, sex, EDSS clinical status, and HLA types of two MS patients (MS-CP and MS-RR) and two healthy controls (NL-sib and NL-match).

proliferation assay using 1–98, 99–172, and 87–106, to determine the region of specificity of each TCL (Voskuhl et al., 1993a).

Statistics

Pearson's χ^2 test with Yate's correction was applied in examining differences between subpopulations of TCLs (Snedecor and Cochran, 1989).

Results

Establishment of X2MBP-specific and 18.5-kDa MBP-specific TCLs by proliferation

We examined the T cell response to X2MBP and 18.5-kDa MBP in three members of a multiplex family and in one unrelated healthy control. When results of the group of four individuals were examined as a whole, without regard to whether TCLs were derived from affected or unaffected, the frequency of TCLs was in agreement with those previously reported for 18.5-kDa MBP (Chou et al., 1989; Richert et al., 1989; Martin et al., 1990, 1992; Ota et al., 1990; Pette et al., 1990; Voskuhl et al., 1993a). Of the 1832 cultures stimulated with 18.5-kDa MBP, the percentage of TCLs positive for 18.5-kDa MBP-specific responses ranged from 7.3% to 11.8% (Table 2). In comparison, frequencies of TCLs specific for X2MBP, a mere 40mer, were of the same order of magnitude as frequencies of TCLs specific for the 172 amino acid 18.5-kDa MBP isoform. Of the 1832 cultures stimulated with X2MBP, the percentage of TCLs positive for X2MBP-specific responses ranged from 0.8% to 13.4%.

Not only was the magnitude of frequencies of TCLs specific for X2MBP as high as 18.5-kDa MBP in the group as a whole, but also the stimulation indices of

X2MBP 40mer containing exon 2 sequence:

PKRGSGK VPWLKPGRSPLP SHARSQPGLCNMYK DSHHPAR

Fig. 3. X2MBP synthetic peptide 40mer containing 26 amino acids encoded by exon 2 (bold type) with overlapping 5' and 3' sequences encoded by exons 1 and 3 respectively.

TABLE 2

Frequency of X2MBP-specific and 18.5-kDa MBP-specific TLCs

	X2MBP-specific			18.5-kDa MBP-specific		
	#pos.	%pos.	est. prec. freq.	#pos.	%pos.	est. prec. freq.
MS-CP	65/484	13.4	6.7×10^{-7}	45/484	9.3	4.6×10^{-7}
MS-RR	16/288	5.6	2.8×10^{-7}	21/288	7.3	3.6×10^{-7}
NL-sib	24/576	4.2	2.1×10^{-7}	50/576	8.7	4.3×10^{-7}
NL-match	4/484	0.8	0.4×10^{-7}	57/484	11.8	5.9×10^{-7}

Comparison of estimated precursor frequencies of X2MBP-specific TCLs with 18.5-kDa MBP-specific TCLs based on stimulation indices (SIs) > 2.

X2MBP-specific TCLs were as high as the stimulation indices of the 18.5-kDa MBP-specific TCLs. A total of 109 X2MBP-specific TCLs were derived from the group as defined by SIs greater than 2 compared to media control and 18.5-kDa MBP control (Table 2, Fig. 4). 94 of 109 (86%) TCLs had an SI greater than 3, and 84 of 109 (77%) had SIs greater than 5. SIs of X2MBP-specific TCLs ranged as high as 786.8:1 when compared to media control and 18.5-kDa MBP antigen control. In comparison, a total of 173 TCLs specific for 18.5-kDa MBP were generated from the same group, as defined by an SI greater than 2 compared to media control and X2MBP control. 132 of 173 (76.3%) TCLs had an SI of greater than 3, and 108 of 173 (62.4%) had SIs greater than 5. SIs for 18.5-kDa MBP-specific TCLs ranged as high as 747.4:1 when compared to media control and X2MBP antigen control. All X2MBP-stimulated and 18.5-kDa MBP-stimulated limiting dilution cultures and proliferation assays were

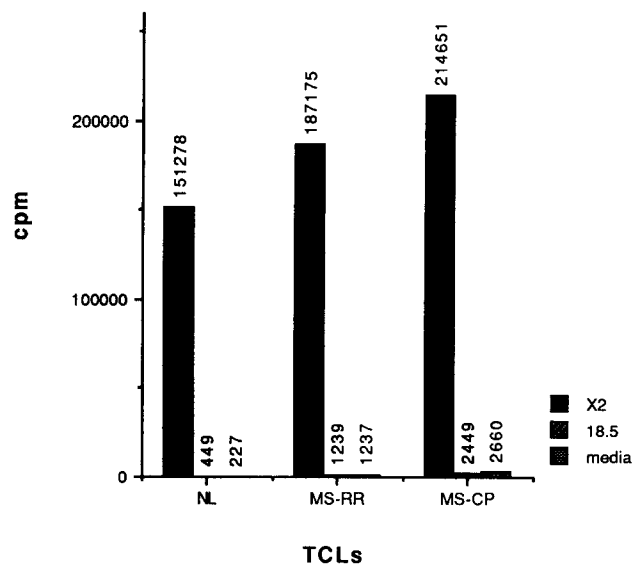


Fig. 4. Proliferative assay results for representative X2MBP-specific TCLs from each member of the multiplex family. Data are expressed as average counts per minute (cpm) from triplicate wells upon stimulation with X2MBP, 18.5-kDa MBP, or media.

carried out in parallel under identical experimental conditions, and reproducibility of frequencies confirmed by repeated experiments.

Comparison of X2MBP-specific responses in affected versus unaffected individuals

From the total of 109 X2MBP-specific TCLs generated, the majority (65%) were derived from the more advanced secondary CP MS patient (MS-CP) (Table 2). From this patient 13.4% of limiting dilution cultures were positive for X2MBP-specific TCLs, resulting in an estimated precursor frequency of 6.7×10^{-7} . This was over twofold higher than the frequency of X2MBP-specific TCLs derived from her mother, the RR patient (MS-RR; 5.6%, 2.8×10^{-7}), $P = 0.0009$, and over threefold higher than that observed in MS-CP's brother, the healthy sibling control (NL-sib; 4.2%, 2.1×10^{-7}), $P < 0.0001$. In comparison with an unrelated age, sex, and HLA class II matched control (NL-match), the frequency of X2MBP-specific TCLs was over 16 fold higher in the secondary CP MS patient (13.4% vs. 0.8%; 6.7×10^{-7} vs. 0.4×10^{-7}), $P < 0.00001$.

There was a slightly higher response to X2MBP in the mildly affected RR MS patient (5.6%, 2.8×10^{-7}) compared to her son, the unaffected related control (4.2%, 2.1×10^{-7}), but this did not reach significance, $P = 0.55$. Each member of the multiplex family including both mildly affected and unaffected demonstrated higher estimated precursor frequencies of X2MBP-specific TCLs when compared to the unrelated healthy control (0.8%, 0.4×10^{-7}), $P < 0.001$.

Comparison of 18.5-kDa MBP-specific responses in affected versus unaffected individuals

In contrast to X2MBP-specific TCLs, the distribution of 18.5-kDa MBP-specific TCLs did not significantly differ between affected and unaffected members of the multiplex family (Table 2), $P = 0.41$. The healthy unrelated control had the highest 18.5-kDa MBP-specific response (11.8% positive, 5.9×10^{-7}) but this did not reach significance, $P = 0.25$, and was largely due to the higher number of relatively lower affinity 18.5-kDa MBP-specific TCLs with SIs greater than 2

TABLE 3

Peptide specificity within 18.5-kDa MBP

Patient	Specificity			
	#TCL	1-98	87-106	99-172
MS-CP	16	3(19%)	2(13%)	11(68%)
MS-RR	12	4(33%)	1(8%)	7(58%)
NL	23	9(39%)	2(9%)	12(52%)

Percentage of 18.5-kDa MBP-specific TCLs derived from each family member that recognize the N-terminal (1-98), the middle (87-106), and the C-terminal (99-172) portion of the 172 amino acid isoform.

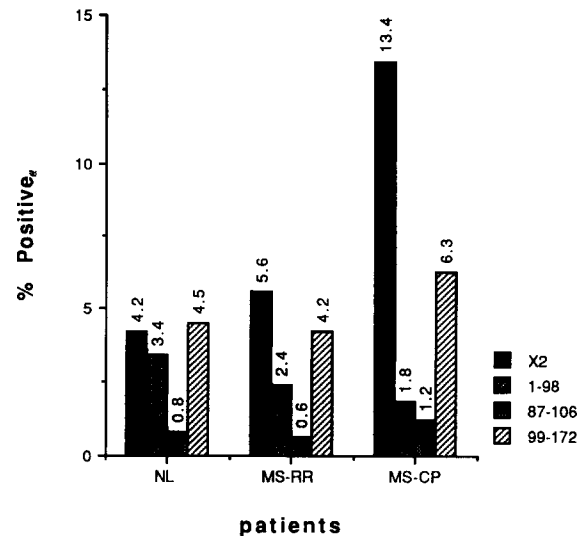


Fig. 5. A comparison of the percentage of cultures specific for X2MBP with those specific for regions containing immunodominant epitopes within the 18.5-kDa MBP isoform; based on the frequency of recognition of 1-98, 87-106, and 99-172 in a representative group of 18.5-kDa MBP-specific TCLs.

but less than 5 (not shown). Further, the N-terminal (1-98), middle (87-106), and C-terminal (99-172) regions were each recognized by TCLs, with similar frequencies between affected and unaffected family members (Table 3).

Comparison of X2MBP-specific responses with those specific for immunodominant epitopes within 18.5-kDa MBP

In each of the multiplex family members the percentage of cultures specific for X2MBP was as high or higher than the percentage of cultures specific for immunodominant epitopes within the N-terminal (1-98), the middle (87-106), or the C-terminal (99-172) regions of 18.5-kDa MBP (Fig. 5).

Discussion

These data confirm our previous observations (Voskuhl et al., 1993b) that in addition to the 18.5-kDa isoform of MBP, distinct epitopes exist in the exon 2 encoded sequence which is contained in the 21.5-kDa and 20.2-kDa isoforms of MBP. Since TCLs specific for X2MBP did not recognize 18.5-kDa MBP (Fig. 4), the epitope recognized by X2MBP-specific TCLs lies within the exon 2 encoded sequence, and is not limited to the terminal overlapping amino acid sequences shared between the X2MBP 40mer and exon 1 or 3 encoded sequences. Also, since the X2MBP epitope is not included in the 18.5-kDa isoform of MBP, T cell responses specific for X2MBP would not have been

detected in previous studies of T cell responses in MS which focused on the 18.5-kDa MBP isoform.

Several epitopes within the 18.5-kDa MBP 172 amino acid molecule have been recognized by TCLs, with immunodominant regions at the N-terminus, the middle, and the C-terminus, with no single epitope having been consistently recognized with greater frequency in MS patients compared to controls (Chou et al., 1989; Richert et al., 1989; Martin et al., 1990, 1992; Ota et al., 1990; Pette et al., 1990; Voskuhl et al., 1993a). This study has recorded similar frequencies of 18.5-kDa MBP-specific TCLs as have been described (Martin et al., 1990; Voskuhl et al., 1993a), and has again found no difference between affected and unaffected members of yet another multiplex family in regard to the frequency of 18.5-kDa MBP-specific TCLs. Again in agreement with the previous study of a multiplex family (Voskuhl et al., 1993a), this family also demonstrated no difference between affected and unaffected in regard to the region of MBP most frequently recognized by TCLs (Table 3).

Within the members of the multiplex family, TCLs specific for X2MBP were surprisingly as prevalent as TCLs specific for multiple epitopes within 18.5-kDa MBP (Table 2). Based upon the frequency of TCLs specific for each region within the 18.5-kDa MBP isoform (Table 3), an epitope, or epitopes, within the 26 amino acid sequence encoded by exon 2 elicited a T cell response as marked as that of immunodominant epitopes within each region of the 18.5-kDa isoform (Fig. 5). In addition, as has been demonstrated for 18.5-kDa MBP-specific TCLs (Martin et al., 1990; Voskuhl et al., 1993a), X2MBP-specific TCLs have been shown to be CD4 + , HLA class II restricted and capable of cytotoxicity (Voskuhl et al., 1993b), thereby demonstrating features characteristic of encephalitogenic MBP-specific T cells which mediate EAE (Powell et al., 1990). Thus, the list of candidate autoantigens in demyelinating/remyelinating autoimmune diseases should be extended beyond assessing T cell reactivity to only the major isoforms of myelin proteins (MBP, PLP, MAG, etc.). T cell reactivity directed toward epitopes unique to isoforms expressed aberrantly during a disease process should also be considered, with X2MBP a leading candidate. In this regard it is of interest that T lymphocytes specific for the gamma subunit of the acetylcholine receptor (AChR) have been detected in patients with myasthenia gravis, since expression of gamma subunit containing AChR is regulated developmentally, occurring primarily in embryonic and also in denervated muscle (Protti et al., 1991).

In this study X2MBP-specific T cell reactivity was investigated in regard to its contribution to the pathogenesis of MS. In order to minimize genetic diversity encountered when studying unrelated patients, members of a multiplex family with MS were studied in an

attempt to identify disease-specific differences in a more restricted MBP response. The frequency of X2MBP-specific TCLs in the more advanced secondary CP patient was significantly higher than the frequency observed in her healthy brother ($P < 0.0001$), as well as in her mother with mild RR disease ($P < 0.001$) (Table 2). Since the secondary CP MS patient did not have the same HLA type as her brother (NL-sib) or her mother (MS-RR), the increased X2MBP-specific response may have been due to HLA differences, as opposed to disease related differences. Therefore, we assessed X2MBP-specific and 18.5-kDa MBP-specific frequencies in an age, sex, and HLA matched healthy control. Class II loci of HLA were chosen for matching since X2MBP-specific and 18.5-kDa MBP-specific TCLs had been previously shown to be CD4 + and HLA class II restricted (Martin et al., 1990; Voskuhl et al., 1993b). In comparing the more advanced CP MS patient with her matched control, the difference in X2MBP-specific reactivity was even greater, $P < 0.00001$. These significant differences in X2MBP responsiveness were very specific for X2MBP and not merely a component of a more generalized difference in immune regulation since no significant difference in reactivity to 18.5-kDa MBP was observed. Thus, within this family the increased T cell response to X2MBP was found in the patient with greatest disability by EDSS (Table 1), and the greatest disease burden by MRI (Fig. 2).

An increased T lymphocyte response specific for X2MBP in more advanced secondary CP patients may contribute to disease through the following mechanism. Remyelination commonly occurs at the edge of MS plaques (Prineas and Connell, 1979; Raine and Scheinberg, 1988), and since MS plaques tend to accumulate in patients with more advanced secondary CP disease, multiple plaques provide multiple foci for remyelination. Since X2MBP expression occurs with myelin formation (Roth et al., 1987; Kamholtz et al., 1988; Kristensson et al., 1986), patients with advanced disease presumably have a higher level of X2MBP expression. X2MBP in areas of remyelination may then serve to drive an ongoing immune response. A cycle of immune mediated demyelination, subsequent remyelination and X2MBP expression, with further immune mediated demyelination may occur in secondary CP MS patients. Thus, the immune response to X2MBP may contribute to the progression of disease in MS from a mild RR pattern to a more advanced secondary CP pattern.

Since X2MBP-specific TCLs were detectable in the peripheral blood of healthy controls, the mere presence of X2MBP-specific TCLs is not sufficient for disease development. In this regard, it is interesting that the healthy member of the multiplex family, had an, albeit less dramatic but still significantly increased ($P < 0.001$), response to X2MBP compared to that of the unrelated healthy control. Disease suscepti-

bility is known to be higher in first degree relatives of MS patients (McFarland et al., 1985; Doolittle et al., 1990) which may be due in part to the coinheritance of susceptible immune response genes, the HLA gene on chromosome #6 and T cell receptor genes on chromosomes #7 and #14 (reviewed in McFarlin and Lachman, 1989). Most recently, polymorphic differences in the region 5' to exon 1 of the MBP gene, #18, have been shown to confer an additional risk for disease susceptibility by both association and linkage analysis (Boylan et al., 1990; Tienari et al., 1992). Although the functional significance of polymorphic differences in the region 5' to exon 1 remains to be established, one could hypothesize that elevated X2MBP-specific responsiveness might reflect inheritance of not only susceptible immune response genes, but also an MBP gene which resulted in altered MBP expression. In this setting of genetic susceptibility, if an initiating event were to induce disease, X2MBP-specific T cells might play a role in disease progression.

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