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T-lymphocyte recognition of a portion of myelin basic protein encoded by an exon expressed during myelination

Rhonda R. Voskuhl, Dale E. McFarlin[†], Roger Stone 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

The major isoform of myelin basic protein (MBP) in the healthy adult central nervous system is the 18.5-kDa protein which is produced by mRNA derived from exons 1, 3, 4, 5, 6 and 7 of the MBP gene. Since isoforms containing exon 2-encoded protein (X2MBP) are expressed during myelin formation, we examined T cell reactivity specific for X2MBP in a disease characterized by remyelination subsequent to demyelination, multiple sclerosis (MS). T cell lines specific for X2MBP were derived from three MS patients as well as one healthy control. This suggests that candidate autoantigens in demyelinating/remyelinating diseases should include not only the major isoforms of myelin proteins, but also isoforms expressed aberrantly during a disease process since they too may be the target of a T cell-mediated autoimmune process.

Introduction

Although myelin basic protein (MBP) is a candidate autoantigen in multiple sclerosis (MS), investigation of T cell responses to the 18.5-kDa isoform of MBP have 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). Characterization of the immune response to other candidate autoantigens in MS has also focused on the major isoforms of myelin proteins expressed in the healthy adult central nervous system (CNS) (Baig et al., 1991; Sun et al., 1991; Trotter et al., 1991). However, several isoforms of myelin proteins exist. Alternative splicing of DNA yields at least two isoforms each for proteolipid protein (PLP), myelin-asso-

ciated glycoprotein (MAG), and 2',3'-cyclic nucleotide 3'-phosphohydrolase (CNP) (Jordan et al., 1989). Four MBP isoforms have been described in humans with molecular masses of 21.5 kDa, 20.2 kDa, 18.5 kDa, and 17.3 kDa (Roth et al., 1987). The healthy adult human CNS is composed primarily of the 18.5-kDa isoform which is encoded by exons 1 and 3 through 7, with a splicing out of exon 2 (Fig. 1). The 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 C57B1/6N 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-encoded protein might be expressed in the adult human CNS during remyelination, and immune reactivity to this protein might exist. This could contribute to the pathogenesis of MS, since remyelination at the edge of MS plaques has been described (Prineas and Connell,

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.

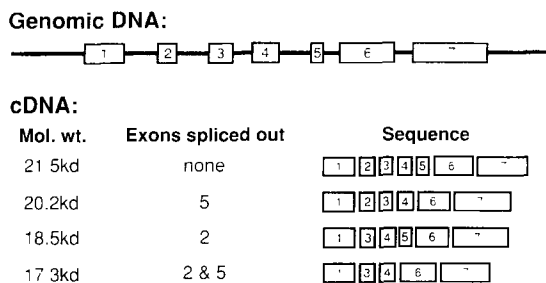


Fig. 1. Germline DNA for MBP gene which yields four cDNA isoforms via alternative splicing (modified from Fritz and McFarlin, 1989).

1979). Consequently, T cell reactivity to exon 2-encoded protein (X2MBP) in the peripheral blood of three MS patients and one healthy control was examined.

Materials and Methods

Patients

Three patients with clinically definite relapsing-remitting MS were studied, as well as one healthy control (Table 1). HLA data for each patient are as listed in Table 1. None were on immunosuppressive medication within 2 years of being studied. All were clinically stable, however one patient (DS), though not in clinical exacerbation, demonstrated gadolinium enhancing lesions on cerebral MRI and mild pleocytosis (9 cells mm⁻³) on cerebrospinal fluid (CSF) examination at the time of study. The research was reviewed and approved by the Institute Clinical Research Subpanel and informed consents were obtained from all patients.

Cells

Peripheral blood lymphocytes (PBL) were obtained by leukapheresis and isolated by sodium-metrizoate density gradient (Martin et al., 1992). Cells were HLA typed by a standard NIH lymphocytotoxicity assay for HLA-A, -B, -C, -DR, and -DQ at the Department of Transfusion Medicine, National Institutes of Health, Bethesda, MD. B cell lines (BCL) were generated from PBL of MS patients and the healthy control by trans-

TABLE 1

Age, sex, EDSS clinical status, disease duration in years (DzDur), and HLA types of three MS patients (DS, MK, DF) and also one healthy control (NL)

	Age	Sex	EDSS	DzDur	HLA-A	HLA-B	HLA-DR	HLA-DQ
DS	23	M	2.0	4	2,32	35,51	4,11	7,-
MK	35	M	4,5	4	2,23	49,8	15,8	6,4
DF	40	F	7,0	15	3,24	7,7	15,15 ^a	6,6 ^a
NL	36	M			1,11	8,27	1,2	1,-

^a Homozygosity confirmed by sequencing.

X2MBP 40mer containing exon 2 sequence:

PKRGSGK **VPWLKPGRSPLPSHARSQPGLCNMYK** DSHHPAR

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

formation with Epstein Barr virus (Jaraquemada et al., 1990).

Antigen

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, our 40-mer protein included sequences to the 5' and 3' regions to provide seven amino acid overlapping regions with exon 1- and 3-encoded protein respectively (Fig. 2). 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).

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

Limiting dilution TCLs were generated from each patient by seeding 2 × 10⁵ PBLs per 200-μl well with X2MBP (10 μg ml⁻¹) in complete culture media. A dose-response proliferative assay confirmed 10 μg ml⁻¹ as the optimal X2MBP concentration (not shown). Cultures using 18.5-kDa MBP (10 μg ml⁻¹) as antigen were generated in parallel for each patient as described (Martin et al., 1992). Each of the limiting dilution cultures was then screened for X2MBP- and 18.5-kDa MBP-specific proliferation (Martin et al., 1992). 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 as X2MBP-specific if they demonstrated an SI > 2 as compared to 18.5-kDa MBP and media controls. Lines were considered positive for 18.5-kDa MBP-specific proliferation if they had SI > 2 as compared to X2MBP and media controls.

Cytotoxic assays

Cell lines positive for X2MBP-specific or 18.5-kDa MBP-specific proliferation were then tested for X2MBP and 18.5-kDa MBP-specific cytotoxic activity by standard chromium release assay (Martin et al.,

TABLE 2
Proliferation assay results for TCLs tested with no antigen (Ag), X2MBP, or 18.5-kDa MBP.

	no Ag ± SE	X2MBP ± SE	SI	18.5kdMBP ± SE	SI
DS-1	11 765 ± 862	118 922 ± 2 096	10.1	10 660 ± 535	0.9
DS-2	976 ± 169	271 064 ± 14 390	277.7	974 ± 204	0.9
DS-3	2 011 ± 484	195 877 ± 10 366	97.4	2 356 ± 467	1.2
DS-4	548 ± 70	153 062 ± 1 332	279.3	681 ± 78	1.2
DS-5	13 924 ± 227	40 713 ± 2 038	2.9	21 828 ± 3 147	1.6
MK-1	4 376 ± 257	60 889 ± 22 767	13.9	5 338 ± 1 234	1.2
DF-1	280 ± 54	1 255 ± 279	4.5	261 ± 34	1.1
DF-2	567 ± 149	2 456 ± 234	4.3	618 ± 67	1.0
NL-1	8 620 ± 905	21 427 ± 1 668	2.5	8 964 ± 586	1.0

Data are expressed as average counts per minute (cpm) from triplicate wells ± standard error (SE) followed by stimulation indices (SI) for each antigen compared to no antigen baseline.

1992). X2MBP-specific or 18.5-kDa MBP-specific proliferative TCLs served as effectors. Targets were autologous EBV-transformed BCL incubated with X2MBP, 18.5-kDa MBP or media alone before being labelled with [⁵¹Cr]sodiumchromate. Cell concentrations were adjusted to reach effector to target (E:T) ratios of at least 20:1 and specific lysis was calculated. TCLs considered positive for X2MBP-specific lysis showed values of 10% or greater as compared to 18.5-kDa pulsed and unpulsed targets. TCL considered positive for 18.5-kDa MBP-specific lysis showed values of 10% or greater as compared to X2MBP pulsed and unpulsed targets.

Phenotyping of cells

The phenotype of TCLs was determined using monoclonal antibodies specific for CD3 (Leu-4), CD4 (Leu-3a) and CD8 (Leu-2a) with mouse IgG₁ control (Becton Dickinson) (Martin et al., 1990).

Statistics

Pearson's X² test with Yates correction was applied in examining differences between subpopulations of TCLs (Snedecor and Cochran, 1989).

TABLE 3
Comparison of estimated precursor frequency of X2MBP-specific TCLs with 18.5-kDa MBP-specific TCLs.

	X2MBP-specific			18.5kd MBP-specific		
	No. TCL pos	%pos	Est. prec. freq. ^a	No. TCL pos.	%pos.	Est. prec. freq. ^a
DS	5/288	1.7	0.9 × 10 ⁻⁷	83/288	28.8	14.0 × 10 ⁻⁷
MK	1/288	0.3	0.2 × 10 ⁻⁷	4/288	1.4	0.7 × 10 ⁻⁷
DF	2/288	0.7	0.3 × 10 ⁻⁷	1/96	1.0	0.5 × 10 ⁻⁷
NL	1/288	0.3	0.2 × 10 ⁻⁷	4/288	1.4	0.7 × 10 ⁻⁷

^a Estimated precursor frequency.

Results

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

Exon 2-encoded protein-specific TCLs were derived from the peripheral blood of three MS patients and one healthy control (Table 2). Stimulation indices of X2MBP compared to media alone ranged as high as 278:1. No lines were identified which responded to both X2MBP and 18.5-kDa MBP with SI > 2 as compared to media alone. The small number of individuals studied precluded analysis of estimated precursor frequency of X2MBP-specific TCLs in MS versus healthy controls. TCLs specific for 18.5-kDa MBP were generated in parallel from the same patients. Stimulation indices of 18.5-kDa MBP compared to media alone ranged as high as 200:1 (data not shown). The estimated precursor frequency of 18.5-kDa MBP-specific TCLs was significantly greater, (*P* < 0.0001), than the frequency of X2MBP-specific TCLs but this result was skewed by the large proportion of 18.5-kDa MBP-specific TCLs from one patient, DS (Table 3). Thus the range of frequency of TCLs specific for X2MBP relative to a previously well characterized antigen (18.5-kDa MBP) run in parallel is demonstrated, however a formal comparison of estimated precursor frequencies for each antigen awaits study of a larger sample size.

Cytotoxicity of X2MBP-specific TCLs

The TCLs shown to be specific for exon 2-encoded protein by proliferation were examined for cytotoxicity. With E:T as low as 10:1, X2MBP-specific TCLs demonstrated specific lysis of X2MBP-pulsed targets (Fig. 3A). X2MBP-specific TCLs did not recognize 18.5-kDa pulsed targets (Fig. 3B). Conversely, 18.5-kDa MBP-specific proliferative TCLs, which had been generated in parallel, demonstrated analogous 18.5-kDa MBP-specific cytolysis (data not shown). Thus X2MBP-specific TCLs demonstrated cytolytic activity similar to the previously reported cytolytic activity of 18.5-kDa MBP-specific TCLs (Martin et al., 1990). Also, similar to 18.5-kDa MBP-specific TCLs, X2MBP-specific TCLs demonstrated HLA class II re-

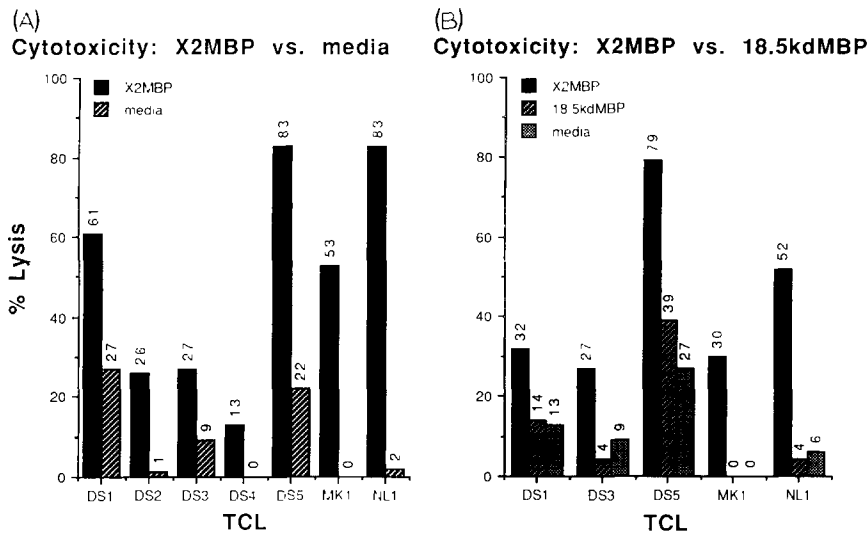


Fig. 3. A. TCLs demonstrating X2MBP-specific cytotoxic activity relative to unpulsed targets. B. TCLs demonstrating X2MBP-specific cytotoxic activity relative to 18.5-kDa MBP pulsed and unpulsed targets.

stricted cytolytic activity, and were primarily CD3 + CD4 + CD8- by FACS analysis (Fig. 4).

Discussion

These data demonstrate that in addition to the 18.5-kDa isoform of MBP, distinct epitopes exist in exon 2-containing isoforms of MBP, 21.5 kDa and 20.2 kDa, which can be recognized by human T cells. Both

X2MBP-specific and 18.5-kDa MBP-specific TCLs were isolated from three MS patients and one control by limiting dilution cultures. TCLs specific for X2MBP did not recognize 18.5-kDa MBP, indicating that the epitope was within the exon 2-encoded sequence, and not limited to the terminal overlapping amino acid sequences shared between the X2MBP 40-mer and exon 1- or 3-encoded sequences. Although the possibility of T cell recognition of a cryptic epitope within the exon 2-encoded protein cannot be excluded without studying responses to 21.5-kDa and 20.2-kDa isoforms, it is noteworthy that the 40-amino acid length of the X2MBP peptide almost certainly requires processing by antigen-presenting cells (APCs).

Since in experimental allergic encephalomyelitis, encephalitogenic MBP-specific T cells which mediate disease are capable of cytotoxicity (Powell et al., 1990), we tested the X2MBP-specific proliferative TCLs for cytotoxicity. Indeed X2MBP-specific cytotoxicity was observed in X2MBP-specific proliferative TCLs with no cross-reactive lysis of 18.5-kDa MBP-pulsed targets. Conversely, the 18.5-kDa MBP-specific proliferative TCLs were cytotoxic for 18.5-kDa MBP-pulsed targets with no lysis of X2MBP-pulsed targets. Therefore, both the X2MBP-specific and 18.5-kDa MBP-specific TCLs are highly specific with no evidence of cross-reactivity. The 18.5-kDa MBP-specific cytotoxic TCLs have been previously described (Martin et al., 1990) while the X2MBP-specific cytotoxic TCLs are a novel finding.

This report is the first to describe TCLs specific for the exon 2-encoded portion of MBP. The only previous description of T cell reactivity specific for a naturally occurring isoform of MBP, other than the cationic 18.5-kDa MBP protein, were TCLs specific for C8, a less cationic 18.5-kDa 172-amino acid protein with citrulline replacing arginine at six residues (Martin et

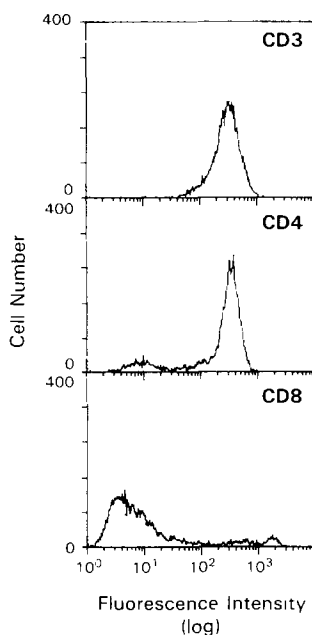


Fig. 4. FACS analysis of CD3, CD4, and CD8 on X2MBP-specific TCLs. 98% of cells stain with high fluorescence intensity using monoclonal antibody (mAb) specific for CD3. 84% of cells were CD4+, while 17% were CD8+. Each mAb was compared to mouse IgG₁ negative control (dotted line).

al., 1991). The demonstration that PBLs from adults contain a population of T lymphocytes which are specific for a myelin protein isoform expressed primarily during developmental myelination and remyelination, with minimal expression in the healthy adult CNS, provides evidence that the list of candidate autoantigens in demyelinating/remyelinating autoimmune diseases may be more extensive than previously anticipated. In addition to assessing T cell reactivity to the major isoforms of MBP, PLP, MAG, etc., isoforms of myelin proteins with increased expression during remyelination should also be considered as targets of a T cell-mediated autoimmune process.

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