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Enhanced T cell responsiveness to citrulline-containing myelin basic protein in multiple sclerosis patients

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Myelin basic protein (MBP), a candidate autoantigen in multiple sclerosis (MS), exists in different isoforms and charge isomers generated by differential splicing of exons and by a combination of posttranslational modifications, respectively. These various isoforms and charge isomers of MBP vary in abundance and most likely serve different functions during myelinogenesis and remyelination. The least cationic among the charge isomers of MBP is citrullinated and is referred to as MBP-C8. MBP-C8 is relatively increased in the population of MBP isomers in more developmentally immature myelin and in MS brain tissue. In a previous study, we found that MBP-C8-reactive T cells could be detected in CD4+ T cell lines (TCL) generated with MBP from both MS patients and normal controls. Here, we examined the frequency and peptide specificity of MBP-C8-specific TCL generated with MBP-C8 in MS patients and controls. Ten subjects grouped in five sets, each an MS patient and a control, were studied. In all cases, the MS patient had either a higher overall number of MBP-C8-responding lines, responded with greater sensitivity to the MBP-C8 antigen or both. Few lines responded to the MBP-C8 peptides but, if they did, they appeared to be specific to the carboxyl-half of the MBP-C8 molecule. Given the large amounts of citrullinated MBP in MS brain tissue, a preferential T cell response to MBP-C8 may be involved in the induction and perpetuation of this disease.
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Introduction

MS, the most frequent inflammatory demyelinating CNS disease in humans, is considered a T cell-mediated autoimmune disease.[1] Its pathogenesis is most likely multifactorial and involves both genetic predisposition and precipitating environmental factors. MBP, which constitutes approximately 30% of the total CNS myelin protein, has been studied extensively as a possible autoantigen in MS.[1] There are four major isoforms of human MBP. The 18.5 kDa isoform is the most abundant in humans.[2] In addition to size heterogeneity, the 18.5 kDa isoform of MBP has at least six charge isomers which can be resolved by cation- or anion exchange chromatography at alkaline pH[3] or according to net positive charge respectively.[4,5] These charge isomers are designated C1, the most cationic, C2–5 which are progressively less cationic by one charge, and C8, the least cationic and the most modified.[5] Posttranslational modifications of the charge isomers include phosphorylation, deamidation, C-terminal arginine loss, and the presence of citrulline substituted for arginine at defined sites in the NH_2- and COOH-terminal portions of the molecule.[5]

In the charge isomer, MBP-C8, six arginines are replaced by citrulline resulting in the loss of six positive charges. In the human MBP molecule of 170 amino acid residues, the arginine to citrulline change occurs on residues 25, 31, 122, 130, 159 and 170.[4] The removal of positive charges alters the interaction of MBP with membrane-embedded acidic lipid moieties.[6] Stoner[7] proposed a model that suggested that certain arginine residues of the MBP molecule stabilize its loop structure by ionic interactions with carboxyl and phosphoryl groups, and that by substitution of citrullines in MBP-C8, a destabilization of the loop structure and possibly a conformational change in the MBP molecule may result. In a study of the formation of myelin crystalline multilayers, Moscarello[8] observed that the denser and less compact myelin showed an increased amount of MBP-C8 and a relative deficiency of MBP-C1. These authors suggest that the more MBP-C1 in myelin, the more compact the myelin layers. Moscarello[9] has also determined that the myelin obtained from MS patients appears to be in a developmentally less mature stage with the percentage of MBP-C8 increased in MS white matter. This MBP-C8-rich, developmentally immature myelin may be

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more susceptible to degradation since it is less compact than mature myelin and cannot form as highly a structured myelin sheath as MBP-C1. The resulting myelin instability may promote myelin breakdown and thus either initiate and/or perpetuate autoimmune T cell responses against CNS myelin.[9] In fact, citrullination of MBP increases the speed of its degradation by cathepsin D.[10] Alternatively, as the number of attacks increase, relatively more MBP-C8 may be formed; MBP-C8 becomes available to the immune system and its increased incidence may perpetuate disease.

The role of MBP is still controversial in MS since most of the evidence in humans is indirect. CD4+ MBP-specific T cells are often cytotoxic,[11,12] directed against immunodominant regions of MBP which are also encephalitogenic in rodent strains,[13–15] often express a Th1-type phenotype,[16] and are frequently restricted by MS-associated HLA-DR molecules.[13,14] Currently, the role of MBP or also other myelin or glial components such as proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG), or αB crystalline, to name only a few, are being evaluated.[1] In addition, other size isoforms and charge isomers of MBP have also been examined both in EAE[17,18] and in MS patients.[19,20] Due to the above reasoning, we have been interested in MBP-C8 as a potential encephalitogen[17] and target antigen in MS.[21] Our data have documented so far that MBP-C8 is indeed able to induce EAE,[17] and a T cell response to MBP-C8 is readily demonstrable in MS patients.[19] The current study extends these previous observations by focusing on the frequencies of MBP-C8-reactive T cells in MS *versus* controls, and also initiates the attempts to characterize the specificity of the C8-reactive lines using a panel of citrullinated MBP peptides.

Material and methods

Patients

Five male patients with definite MS, four with primary progressive (PP), one with relapsing-remitting (RR) MS, were investigated at the Neuroimmunology Branch, NINDS, National Institutes of Health (NIH). We attempted to examine patients with progressive disease since an accumulation of MBP-C8 in MS brains with increasing disease duration had been reported.[9] The research project was reviewed and approved by the Institute Clinical Research Subpanel, and informed consent was obtained from each patient. MS patient demographics and clinical data are summarized in Table 1. No patient was or had been on immunomodulatory or immune suppressive treatment at any time or had received glucocorticoids within 90 days.

Cells

Peripheral blood lymphocytes (PBL) were isolated from leukapheresis samples by Lymphocyte Separation Medium (Organon Teknika, Durham, NC, USA). After isolation, cells were cryopreserved in RPMI 1640 (Whittaker Bioproducts, Walkersville, MD, USA) supplemented with 20% fetal calf serum (GIBCO, Grand Island, NY, USA) or 20% human serum, and 10% dimethylsulfoxide (Sigma, St. Louis, MO, USA) and stored in liquid nitrogen until use. HLA typing for HLA-A, -B, -Cw, -DR, and -DQ was performed kindly by T Simonis, Department of Transfusion Medicine, NIH, Bethesda, MD, USA. HLA-DR and -DQ types are summarized in Table 1.

Antigens

MBP-C1 was prepared from postmortem human brain.[22,23] MBP-C8 was prepared from the pH3 extract of human brain by a combination of cation-exchange chromotography and high-pressure liquid chromatography (HPLC) as described.[4] Citrulline-containing MBP-peptides were made for regions containing citrulline modifications.[19] Using a numbering system based on 170 residues in MBP, the following three citrulline-containing peptides were synthesized as described:[24] 18–38 (ASTMDHA**Cit**HGFLP**Cit**HRDTGIL) with citrulline substitutions at positions 25 and 31, 115–131 (SWGAEGQ**Cit**PGFGYGG**Cit**A) with citrulline substitutions at positions 122 and 130, and 151–170 (SKIFKLGG**Cit**DSRSGSPMARR) with a citrulline substitution at position 159. No posttranslational modifications other than citrullination were included in the peptide sequences. All peptides were purified by using HPLC to a purity >95%.

Table 1 Summary of clinical features and HLA haplotypes of MS patients

	MS1	*MS2*	*MS3*	*MS4*	*MS5*
Sex	M	M	M	M	M
Age	43	59	49	67	49
MS type	PP[a]	PP	RR[b]	PP	PP
Disease	SC[c]:1980	SX:1965	SX:1981	SX:1959	SX:1979
Suration	DX[d]:1988	DX:1974	DX:1988	DX:1965	DX:1984
Disability[e]	8.0	8.5	3.0	7.0	7.5
HLA-DR	B1*0101 B1*0405	B1*0402 B1*1502	2(15),4	2	2(15),4

[a]PP=primary progressive. [b]RR=relapsing remitting. [c]SX=onset of symptoms. [d]DX=year of diagnosis. [e]Disability expressed on Expanded Disability Status Scale (Kurtzke, 1983)

Generation of MBP-C8-specific T cell lines (TCL)
PBL were seeded in complete media (Iscove's modified Dulbecco's medium with 2 mM L-glutamine, 50 μg/ml gentamicin, 100 U/ml penicillin, and 100 μg/ml streptomycin-Whittaker Bioproducts, Gaithersburg, MD, USA) at 2×10^5 cells/well into 96-well, U-bottom microtiter plates (Nunc, Denmark) at 200 μl per well and stimulated with 5 μg/ml of MBP-C8. Seven to 10 days later, IL-2 (Lymphocult-T HP, Biotest, Germany; final concentration, 10 U/ml) was added. After another 7 to 10 days, cells were washed once within the plate to remove lymphokines and resuspended in 200 μl of complete medium. Fifty μl of the cell suspension were transferred into three adjacent wells of a separate 96-well, U-bottom microtiter plate, and 150 μl of complete medium containing 1×10^5 autologous, irradiated (3000 rad) PBL added. One well was stimulated with 5 μg/ml MBP-C8 and the other with 5 μg/ml MBP-C1. Seventy-two hours later, 1 μCi of [^{3}H]thymidine (Amersham, Arlington Heights, IL, USA) was added for 4 h before [^{3}H]thymidine incorporation was measured in a scintillation counter (Betaplate, Pharmacia, Gaithersburg, MD, USA). The remaining wells in the original plates were restimulated with 1×10^5 autologous, irradiated PBL and MBP-C8 at 5 μg/ml at the same time the proliferation was analyzed. Wells with a stimulation index (SI=c.p.m. of wells with antigen/c.p.m. of wells without antigen) >2 and >1000 c.p.m. in the proliferative assay were transferred into 24-well plates (Costar, Cambridge, MA, USA), restimulated as described above, and characterized for specificity by proliferative assay.

Proliferative assays
Proliferative assays were performed as described.[11] Briefly, 2×10^4 line cells were incubated for 72 h with $5-10\times10^4$ irradiated (3000 rad), autologous PBL as antigen-presenting cells (APC) and two concentrations (10 and 20 μg/ml final concentration) of antigens or peptides. One μCi of [^{3}H]thymidine (Amersham) was added for another 4 h. Thymidine incorporation was measured in a scintillation counter (Betaplate, Pharmacia, Gaithersburg, MD, USA). Responses were scored positive at an SI>2 and >1000 c.p.m.

Results

CD4+ TCL were generated with MBP-C8 by seeding in parallel PBL from five MS patients and five normal controls in a standard split well limiting dilution assay. During the split-well screening, which is routinely performed between 14 and 17 days after seeding, colonies are tested against MBP-C1, MBP-C8 and no antigen. Responses were scored positive at an SI>2 and >1000 c.p.m. The TCL were grouped in three categories based on their proliferative responses to the antigens: MBP-C8-responsive only, MBP-C1-responsive only as well as MBP-C8/MBP-C1-responsive. As shown in Table 2, patient/control set number 1 showed the most impressive differences in the C8

Table 2 Summary of frequency and specificity of MBP-C8 responsive T cell lines from MS patients and normals

				Antigen							
				MBP-C1/MBP-C8							
				C1		*C8*		*MBP-C8 only*		*MBP-C1 only*	
Sets	*Patients*	*Normal*	*SI*	*M*	#	*M*	#	*M*	#	*M*	#
1	MS1		$\geqslant3$	6.0	12	7.9	20	4.7	7	4.8	1
			$\geqslant5$	7.4	7	8.9	16	8.2	2	0	0
		ND1	$\geqslant3$	4.3	1	4.4	1	0	0	0	0
			$\geqslant5$	0	0	0	0	0	0	0	0
2	MS2		$\geqslant3$	17.4	2	20.7	2	16.1	6	3.1	1
			$\geqslant5$	29.8	1	20.7	2	22.3	4	0	0
		ND2	$\geqslant3$	13.5	7	12.5	9	5.8	3	4.0	1
			$\geqslant5$	16.7	5	26.4	5	5.8	3	0	0
3	MS3		$\geqslant3$	6.1	10	5.1	9	6.2	3	4.9	5
			$\geqslant5$	11.4	3	6.9	4	7.7	2	6.2	3
		ND3	$\geqslant3$	4.6	4	4.1	3	0	0	0	0
			$\geqslant5$	7.8	1	5.0	1	0	0	0	0
4	MS4		$\geqslant3$	7.1	3	11.7	3	0	0	0	0
			$\geqslant5$	7.1	3	11.7	3	0	0	0	0
		ND4	$\geqslant3$	0	0	0	0	0	0	3.1	3
			$\geqslant5$	0	0	0	0	0	0	0	0
5	MS5		$\geqslant3$	193.3	2	81.5	3	5.4	2	0	0
			$\geqslant5$	193.3	2	120.7	2	7.2	1	0	0
		ND5	$\geqslant3$	6.2	8	6.4	8	9.6	1	0	0
			$\geqslant5$	8.5	5	7.8	5	9.6	1	0	0

SI: stimulation index; M: mean value of the stimulation indices; #: number of positive lines generated

response between the MS patient and the normal donor. Sets 1 through 4 demonstrated a higher percentage of MBP-C8-reactive lines in the MS patients compared to the normal controls. In one normal donor, number 5 (ND5), one more MBP-C8 only-reactive line was established compared to the patient MS5. However, the stimulation indices for the patient's MBP-C8 lines (both the MBP-C8/MBP-C1 and the MBP-C8 only lines) had a mean value of 32.8 while the mean value for the normal control was only 4.8. Thus, although one more TCL had been generated from donor ND5, their reactivities were not as strong as those from the MS patient. Sets 1, 3 and 4 demonstrated a higher percentage of MBP-C8/MBP-C1-reactive lines in the MS patients than in the normal controls. However, in sets 2 and 4, the lines that responded to both MBP-C1 and MBP-C8/MBP-C1 were more numerous in the controls than in the patients.

Statistical analysis (*t*-test) was performed between the TCL derived from MS patients and controls at each cut-off value (SI greater than or equal to 3 and 5) for the total number and the mean SI value of the three groups of TCL: TCL responding to MBP-C8/MBP-C1, to MBP-C8 only, or to MBP-C1 only. Further analysis was performed to analyze the differences in the total number of MBP-C8-reactive lines between MS patients and controls. In each group, the mean number of MBP-C8-responding lines in the MS patients was higher (in many cases as much as three times higher) than the mean for the controls both with respect to mean SI and number of responding lines. However, due to the limited number of data sets, our sample size was too small to reach statistical significance, but remained a trend.

In order to test whether the trend towards a stronger MBP-C8-specific response was also reflected in the magnitude of the proliferative response of individual lines, we analyzed the data from these 10 individuals with respect to the stimulation indices for the positive lines. The number of positive lines for each individual was determined using more stringent criteria, i.e. cut-off values for the SI of greater or equal to 3 and then again with a value of greater or equal to 5. In sets 1, 2, 3 and 5, the numbers of MBP-C8 only-reactive lines were higher in the MS patients than in controls at both cut-off levels. Set 4 (both patient and control) failed to show any MBP-C8 only-reactive lines at the >3 or >5 SI cut-off values. Using these criteria for the MBP-C8/MBP-C1 lines, sets 1, 3 and 4 demonstrated a higher number of positive lines at both SI values in the patients as compared to the controls. In contrast, sets 2 and 5 had higher numbers of positive lines at both SI cut-off values in the normal individuals as compared to the MS patients. There is not only a trend towards higher numbers of MBP-C8-responding lines in MS patients *versus* controls, but these also exhibited higher SIs than those of the control individuals.

A large proportion of the highly MBP-C8-responsive lines remained antigen-specific upon confirmatory testing (Figure 1) using the original MBP-C8 antigen.

In an attempt to map the epitope specificity of MBP-C8-specific TCL to individual citrulline residues, the MBP-C8-positive lines were tested against a panel of three MBP-C8 peptides (Figure 2) corresponding to the three regions of MBP where the citrulline modifications occur. Of the 11 MS1 MBP-C8-positive lines tested against whole MBP-C8, MBP-C1, and the three MBP-C8 peptides, all 11 recognized whole MBP-C8, 10 recognized MBP-C1 and three recognized MBP-C8 peptides. As shown in Figure 2, TCL 1C8 gave an SI of 21.3 for whole MBP-C8, an SI of 2.1 for MBP-C1, and an SI of 19.5 for MBP-C8 peptide 151–170. MS1 TCL 3A5 responded with an SI of 114.3 to MBP-C8, an SI of 29.1 for MBP-C1, and an SI of 75.2 for MBP-C8 peptide 151–170. MS1 line 2A4 responded with an SI of 103.9 to MBP-C8, 150.0 to MBP-C1, and 2.2 to MBP-

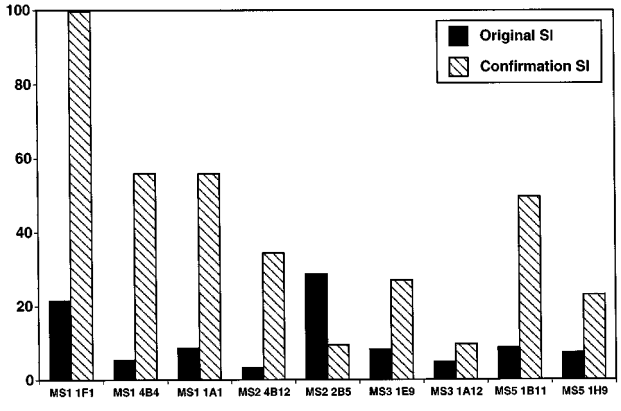

Figure 1 *Confirmatory testing for antigen-specific response of MBP-C8-elicited T cell lines. The MBP-C8-responsive T cell lines were generated as described in Materials and methods section. The proliferative responses to MBP-C8 of these T cell lines at short term (after three cycles of antigen stimulation) and long term (after more than five cycles of antigen stimulation) were compared*

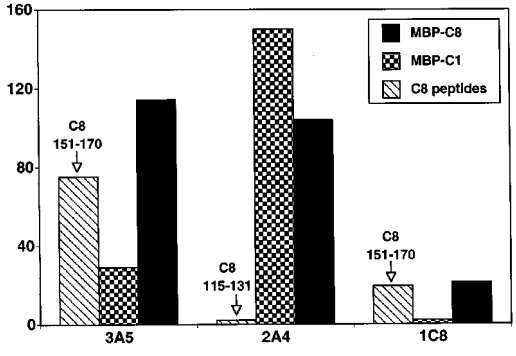

Figure 2 *Proliferation assay for T cell response to MBP isomers and MBP-C8 peptide. Eleven MBP-C8 generated T cell lines from two MS patients were examined for their responses to MBP-C8 peptides. The heterogeneity in epitope specificity among these T cell lines was evident and three typical patterns of epitope recognition are shown in the figure*

C8 peptide 115–131. Further attempts to map the peptide specificity to individual citrulline residues were not successful.

Discussion

Many potential candidate autoantigens, particularly MBP, PLP and MOG, have been studied for their relevance in MS and encephalitogenicity in EAE models.[1] Assessing biological relevance in humans has to rely on indirect evidence such as increases in T cell frequency, higher affinity for the antigen, memory phenotype, cytokine profile, i.e. secretion of proinflammatory cytokines, and cytotoxicity. The results of the present study demonstrate that the T cell response against a post-translational modification of MBP, i.e. MBP-C8, is relatively enhanced in MS patients as compared with HLA-DR-matched control donors. Due to the limited availability of MBP-C8, a larger cohort of MS patients could not be studied, and it needs to be stressed that our current results have been obtained with MS patients with long-standing disease and an overrepresentation of PP-MS. The increased response in MS patients is evident in some cases as a higher total number of MBP-C8-reactive TCL and, in other cases, as higher stimulation indices in the reactive lines or both. In addition, they were tested for reactivity against three citrullinated peptides of MBP. TCLs derived from patient MS1 showed a predominant reactivity to the C-terminal MBP-C8 peptide 151–170. We were unable to map further the fine specificity in the other two MBP-C8 peptides. Furthermore, our overall limited success with determining the peptide specificity may indicate either that the MBP-C8 isolated from native brain myelin contains other important post-translational modifications which were not present in the synthetic, citrulline-containing peptides, or that the natural proteolytic cleavage of MBP-C8 in antigen-presenting cells creates different peptides than the ones chosen for synthesis.

The relatively higher level of MBP-C8 in MS brain[9] could help explain our finding of a prominent role for MBP-C8 as an antigen for T cells in MS. The continuous or repeated release of MBP-C8 and its *de novo* synthesis could lead to an *in vivo* expansion of MBP-C8-specific T cells. The trend for an overall greater response to MBP-C8 in MS patients (with respect to both number of MBP-C8 responsive TCL and magnitude of their SIs) supports these considerations.

Besides an overall stronger response to MBP-C8 in MS patients, it is also noteworthy that the MBP-C8-specific response exceeded that to MBP-C1 considerably, e.g. in patient MS1. Whether this is due to a higher abundance of MBP-C8 in this patient or higher immunogenicity of MBP-C8 for T cells in this individual is unresolved. The latter possibility may be due, in part, to the fact that certain native MBP isoforms are expressed in the thymus where tolerance may be established.[25] It is not known if central, i.e. thymic, tolerance to the developmentally more immature MBP-C8, occurs.

Similar to other isoforms of MBP, e.g. the 21.5 kd MBP in which exon 2 is expressed, MBP-C8 may be relevant to disease pathogenesis not only since its expression is upregulated during the disease process, but also due to the physicochemical instability of MBP-C8 compared to the more basic MBP charge isomers. In our previous report,[10] under the condition of enzymatic degradation, MBP-C8 is degraded much faster by cathepsin D than MBP-C1 to release immunodominant epitopes which presumably prime not only MBP-C8-reactive T cells but also MBP-C1-responsive T cells. This was proved by the findings of the current experiment in which some human T cell lines generated with MBP-C8 react with both MBP-C8 and MBP-C1 and to our surprise, some even respond to MBP-C1 only. With respect to MBP-C1-only reactive lines, it is most likely that the absence of reactivity to MBP-C8 which had been used to generate the lines initially, is due to differences in the relative sensitivity towards the two antigens, i.e. MBP-C1 induces a heteroclitic response, causing stimulation at lower antigen doses compared to MBP-C8 as previously reported.[26] In two of three multiplex families studied there was a significantly increased exon 2 MBP-specific response even though there was no difference in the frequency of exon 2 MBP-specific T-cell between the MS group and the healthy control group.[20]

The T cell response to the MBP-C8 isomer is elevated in MS patients, but clearly also, though in a smaller scale, present in healthy controls. Future studies will need to address whether increased expression and immunoreactivity to MBP-C8 correlates with specific disease courses and subtypes of MS in larger groups of patients stratified for these criteria. In addition, cytokine profiles of the MBP-C8 T cell lines should be examined in the future to assess for differences between the MS patients and the normal controls. The encephalitogenicity of MBP-C8, its structural instability and the results of the present investigation underscore the prominent role for a selective charge isomer of MBP. Other size isomers and post-translational modifications of MBP may deserve more detailed analysis for their roles in the autoimmune response to MBP.

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