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## Peptides of myelin basic protein stimulate T lymphocytes from patients with multiple sclerosis

Constantin N. Baxevanis<sup>1</sup>, George J. Reclos<sup>1</sup>, Catherine Servis<sup>3</sup>, Emmanuel Anastasopoulos<sup>1</sup>,  
Paula Arsenis<sup>1</sup>, Anna Katsiyiannis<sup>1</sup>, Nikolaos Matikas<sup>2</sup>,  
John D. Lambris<sup>3</sup> and Michael Papamichail<sup>1</sup>

<sup>1</sup> Department of Immunology, Hellenic Anticancer Institute, Athens, Greece,

<sup>2</sup> Neurology Department, Evangelismos Hospital, Athens, Greece, and <sup>3</sup> Basel Institute for Immunology, Basel, Switzerland

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### Summary

Peripheral blood T lymphocytes from patients with multiple sclerosis (MS) and other neurological diseases (OND) were tested for primary *in vitro* proliferation in response to four synthetic peptides derived from the sequence of human myelin basic protein (HuMBP) and to HuMBP 45–89 peptide fragment, using a [<sup>3</sup>H]thymidine incorporation assay. The synthetic peptides used corresponded to residues HuMBP 15–31, 75–96, 83–96 and 131–141 of human myelin basic protein. Significant proliferation of T lymphocytes to peptides was noted only in the MS group (with the exception of peptide 131–141); the majority of control subjects and OND patients did not respond to the above-mentioned peptides. The sensitized T lymphocytes in MS patients displayed the inducer/helper phenotype and required autologous monocytes for optimal proliferation. An anti-HLA-DR monoclonal antibody, directed against a monomorphic determinant of DR molecules, was able to block the responses in a dose-dependent fashion. These results suggest that autoimmune inducer/helper T lymphocytes in the peripheral blood of MS patients may initiate and/or regulate the demyelination process in patients with MS. Furthermore, our data demonstrate that monocytes and HLA-DR molecules are essential for activation of these cells. Finally primary *in vitro* T cell proliferation to HuMBP synthetic peptide may be used as an additional diagnostic test in MS.

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Address for correspondence: Constantin N. Baxevanis, Ph.D., Department of Immunology, Hellenic Anticancer Institute, 171 Alexandras Ave., 11522 Athens, Greece.

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### Introduction

Human myelin basic protein (HuMBP) is composed of 170 amino acid residues, has a monomeric molecular weight of 18 500, and accounts for nearly one-third of the protein in central nervous system (CNS) myelin (Norton, 1981). A

great deal of interest has centered on MBP, as it has been shown to be the encephalitogenic determinant for producing experimental allergic encephalomyelitis (EAE) in animals (Carnegie and Moore, 1980; Pettinelli et al., 1982). In Lewis rats, the major encephalitogenic region resides within the 68–88 amino acid sequence of guinea pig MBP or rat MBP (Chou et al., 1977) whereas synthetic polypeptides of the N-terminal sequence (residues 1–16) of mouse MBP were found to cause EAE in mice (Zamvil et al., 1986). In relation to MBP fragments present *in vivo*, evidence indicates that MBP peptide 45–89 and fragments thereof, particularly residues 80–89, constitute the major antigenic portion of MBP in cerebrospinal fluid (CSF) after acute CNS myelin injury (Whitaker and Gupta, 1985). Recently, Price et al. (1987) produced monoclonal antibodies specific for several synthetic peptides of the HuMBP 45–89 sequence, demonstrating the multiplicity of immunogenic epitopes included in this antigenic region.

The presence of a marked inflammatory response associated with the demyelination has been considered as evidence that multiple sclerosis (MS) may be a cell-mediated autoimmune disease (McFarlin and McFarland, 1982; Waksman and Reynolds, 1984). T cell sensitivity to MBP has been demonstrated in MS patients in both CSF and peripheral blood (Sheremata et al., 1974; Knight, 1977; Lisak and Zweiman, 1977; Lisak et al., 1984). To focus on possible epitopes on MBP recognized by T lymphocytes, we used synthetic MBP peptides to trigger human T cell proliferative responses *in vitro*.

We found that synthetic peptides of the HuMBP sequences 15–31, 75–96, 83–96 and HuMBP fragment 45–89 stimulated peripheral blood T lymphocytes from MS patients (but not from normal donors or other neurological diseases (OND) patients) to proliferate in primary cultures. This finding demonstrates the presence of autoimmune (memory) T lymphocytes specific for MBP fragments in the peripheral blood of MS patients. In addition it shows that regions other than HuMBP 45–89 can also be immunogenic. The requirements for T cell proliferation to HuMBP peptides in MS patients were also studied and it was found that both autologous monocytes and HLA-DR antigens (expressed on monocytes) are important

in the pathway leading to HuMBP-induced activation of T lymphocytes from patients with MS.

## Materials and methods

### *Preparation and synthesis of peptides*

HuMBP peptide 45–89 was isolated and characterized as described (Chou et al., 1976; Whitaker and Seyer, 1979). Synthetic peptides used in this study are listed in Table 1 along with their sequences and molecular weights. All peptides were synthesized using an Applied Biosystems Model 420A automated peptide synthesizer. The peptides were synthesized on a *para*-methylbenzyl-hydroxylamine resin, using *t*-Boc-L-protected amino acids (Bachem). The coupling was performed in dimethylformamide (DMF)/dichloromethane (DCM) using dextran-coated charcoal (DCC) as a coupling agent followed by neutralization with *N,N*-diisopropylethylamine (DIEA). The coupling efficiency was followed after each cycle by the Kaiser-Ninhydrin test (Kaiser et al., 1970). The final product was cleared from the resin using high anhydrous hydrofluoride (HF). Purification of the peptides was accomplished by high performance liquid chromatography (HPLC) on a reversed phase C18 (Vydac) column using a 40 min gradient of (1–100%) acetonitrile containing 0.1% trifluoroacetic acid (TFA). The peptide 75–96 was synthesized with an inverted Gly–His at positions 77–78 as suggested by Price et al. (1986).

### *Cell preparation*

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood by

TABLE 1  
AMINO ACID SEQUENCES OF THE SYNTHETIC PEPTIDES USED IN THIS STUDY

Peptide residue	Sequences <sup>a</sup>	Da
15–31	LATASTMDHARHGFLPR	1881
75–96	KSHGRTQDENPVVHFFKNIIVTP	2982
83–96	ENPVVHFFKNIIVTP	1915
131–141	ASDYKSAHKGF	1303

<sup>a</sup> Amino acid sequence is taken from Carnegie (1971).

means of Ficoll-Hypaque gradient density centrifugation (Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.). Monocytes (>90% positive with anti-Leu-M1, anti-Leu-M3 and anti-Leu-M5 monocyte-specific monoclonal antibodies) were isolated by adherence on plastic pretreated with serum (Baxevanis et al., 1987a). T lymphocytes (>95% positive with anti-Leu-4 T cell-specific monoclonal antibody) were isolated after passing PBMC through nylon-wool columns (Julius et al., 1973). To prepare T cell subsets a method analogous to one described by Thomas et al. (1980) was used. Briefly,  $20 \times 10^6$  T lymphocytes/ml in culture medium were incubated with OKT4 or OKT8 monoclonal antibodies (1:40 final dilution) for 1 h at room temperature followed by 1 h incubation at 37°C with rabbit complement (RC') (Cappel Laboratories, Cochranville, PA, U.S.A.) at a final dilution of 1:12. To assess the completeness of killing, the residual cells were analyzed using the anti-Leu-2a and anti-Leu-3a reagents, which identify the same T cell subsets as OKT8 and OKT4 monoclonal antibodies respectively (Ledbetter et al., 1981). Cells remaining after treatment with OKT8 + RC' ( $T_4^+$  cells) were 96% Leu-3a<sup>+</sup>; treatment with OKT4 + RC' yielded 93% Leu-2a<sup>+</sup> cells ( $T_8^+$  cells). All monoclonal antibodies were purchased from Becton and Dickinson (Mountain View, CA, U.S.A.) and cells were analyzed by indirect immunofluorescence (Baxevanis et al., 1987b; Reclos et al., 1987). Anti-HLA-DR monoclonal antibody L243 was kindly provided by Dr. D.S. Monos, Department of Pathology, Hospital of the University of Pennsylvania, Philadelphia, PA, U.S.A. This antibody is specific for a monomorphic determinant of HLA-DR molecules (Schackelford et al., 1981).

#### *Peptide-pulsing of monocytes*

This was performed as previously described (Baxevanis et al., 1988). Monocytes ( $1 \times 10^6$  cells/ml) were incubated for 1 h at 37°C in RPMI-1640 culture medium (Gibco, Grand Island, NY, U.S.A.) (supplemented with 10% pooled, heat-inactivated human AB serum, antibiotics and L-glutamine) in the presence of 100 µg/ml of the peptides and 50 µg/ml mitomycin C (Kyowa Hakko, Tokyo, Japan). Control (non-pulsed) monocytes were treated with mitomycin C only.

#### *Cell proliferation analysis*

T lymphocytes or T lymphocyte subsets ( $4 \times 10^5$  cells/well) were incubated in 0.2 ml of culture medium with peptide-pulsed or control (non-pulsed) autologous monocytes in microculture plates (Greiner, Kirchheim, u.Teck, Stuttgart, F.R.G.) at 37°C in a moist atmosphere of 5% CO<sub>2</sub> in air. Cultures were pulsed for 16 h with 1.0 µCi [<sup>3</sup>H]thymidine (Amersham) prior to harvesting in an automatic Dynatech Cell Harvester (Dynatech Products, Switzerland). Stimulation data is presented as mean cpm incorporated and/or stimulation index (SI).  $SI = [(cpm \text{ incorporated in peptide-stimulated cells (test cultures)}) / (cpm \text{ incorporated in unstimulated cells (background cultures)})]$ . In some experiments T lymphocytes were cultured with non-pulsed autologous monocytes as described above in the presence of 100 µg/ml of HuMBP peptides (20 µg/well).

#### *Patients*

Twenty-five patients (ten male, 15 female) with MS were studied. All patients had severe progressive disease with worsening in the 9 months before entry. Worsening, defined as a decrease in one or more points on the functional status or disability scale, consisted of either a continuous decline or a continuous decline with superimposed exacerbations. Eighteen patients were ambulatory, and seven had first required the use of a wheelchair within 3 months of entry into the study. The control populations consisted of 25 healthy individuals and 25 with other neurological diseases including amyotrophic lateral sclerosis (ALS), acute inflammatory polyneuropathy (AIP), Parkinson's disease, myasthenia gravis (MG) and cerebrovascular accidents. No patients or healthy donors received steroids or other immunosuppressive agents during the month before lymphocyte analysis, and none had a second major illness.

#### **Results**

The proliferative response of peripheral blood T lymphocytes to HuMBP 45–89 peptide fragments and HuMBP 15–31, 75–96, 83–96 and 131–141 synthetic peptides is shown in Fig. 1 and summarized in Fig. 2. The distribution of the

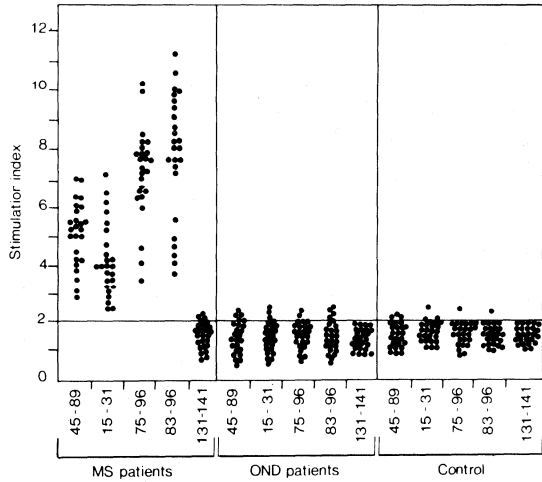


Fig. 1. The proliferative response of peripheral blood T lymphocytes to HuMBP peptides. Each point represents a single individual. Significant proliferation (compared to background proliferation) is considered above SI = 2.

proliferative response is shown in Fig. 1. It is evident from Fig. 2 that MS patients had a significantly higher proliferative response compared to patients with OND ( $P < 10^{-3}$ ) or to healthy individuals ( $P < 10^{-3}$ ). Even when the subjects were considered individually, it is also clear that MS patients were definite responders to HuMBP peptides, with the exception of peptide 131-141.

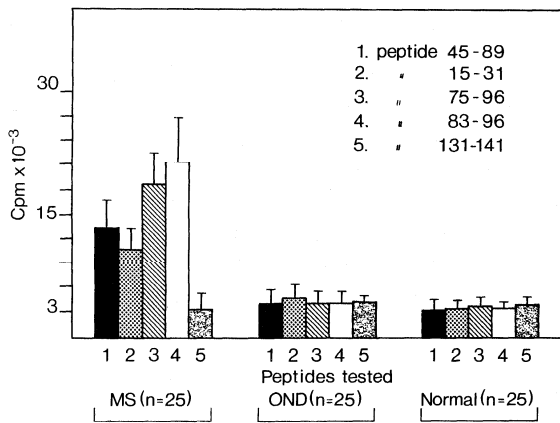


Fig. 2. Proliferation of peripheral blood T lymphocytes to HuMBP peptides. Values represent the mean cpm  $\pm$  SD from the pooled data. The molarity of HuMBP peptides used was for synthetic peptides 15-31:  $5.3 \times 10^{-5}$  M; 75-96:  $3.35 \times 10^{-5}$  M; 83-96:  $5.22 \times 10^{-5}$  M; 131-141:  $7.6 \times 10^{-5}$  M; fragment 45-89 (MW 4886):  $2.04 \times 10^{-5}$  M.

The majority of patients with OND and healthy individuals did not respond significantly ( $P > 0.5$ ;  $SI < 2$ ) to these peptides. However, there were some exceptions where a minor, although significant, response ( $P < 0.05$ ;  $SI \geq 2$ ) compared to the background, was noticed. Three out of 25 patients with OND (12%) responded to HuMBP 45-89 (ALS; MG) and 3 (12%) responded to HuMBP 15-31 (AIP). Two OND patients (8%) responded to HuMBP 75-96 (Parkinson's disease and MG) and four patients (16%) responded to HuMBP 83-96 (Parkinson's disease; ALS; MG). Among the healthy donors, three out of 25 (12%) responded to HuMBP 45-89, only two responded to HuMBP 15-31, one person responded to HuMBP 75-96 and one to HuMBP 83-96. There was no significant response induced by HuMBP 131-141 except in two out of 25 (8%) MS patients ( $P < 0.05$ ). The magnitude of the responses in the MS group (with the exception of the anti-HuMBP 131-141 responses) was considerably higher compared to the significant ones in the OND group and the group of healthy subjects (SI range 3-11, except in five cases where the SI was between 2 and 3).

As shown in Fig. 3 the absence of monocytes from the cultures virtually eliminated responses to HuMBP peptides. Moreover, a monoclonal anti-HLA-DR antibody (L243) when added in cultures

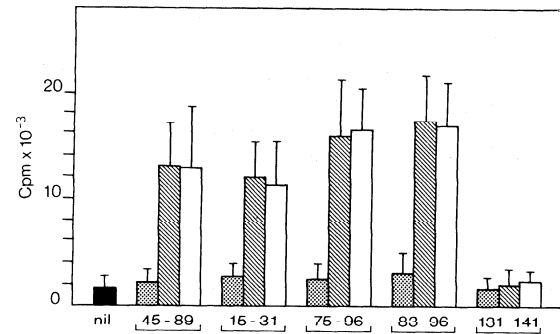


Fig. 3. Peripheral blood T lymphocytes from MS patients ( $n = 10$ ) were tested for proliferative responses to HuMBP peptides in the presence of the peptides alone (stippled bars), or with peptide-pulsed autologous monocytes (hatched bars) or with non-pulsed autologous monocytes in the presence of the peptides (open bars). Background groups consisted of T lymphocytes and autologous non-pulsed monocytes (closed bars). Data are given as mean cpm  $\pm$  SD of the pooled data.

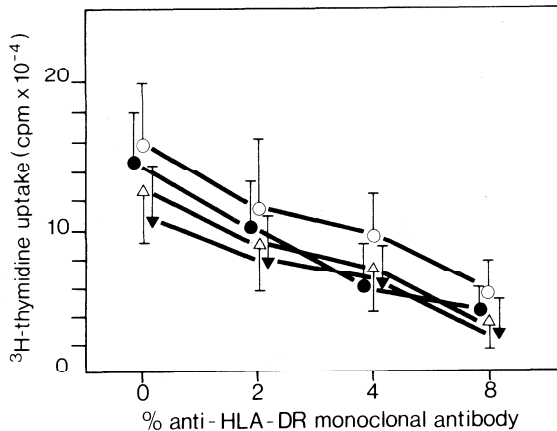


Fig. 4. Inhibition of HuMBP peptide-induced T lymphocyte proliferation by anti-HLA-DR monoclonal antibody (added at the initiation of the culture). HuMBP 83-96 (○); HuMBP 75-96 (●); HuMBP 45-89 (△); HuMBP 15-31 (▼). Pooled data (eight MS patients were tested) are expressed as mean  $\text{cpm} \pm \text{SD}$ . Percentage of L243 antibody was calculated in 200  $\mu\text{l}$  total volume per culture.

could highly block T lymphocyte proliferation (Fig. 4). These data suggest that monocytes are necessary, as antigen-presenting cells, in order to stimulate HLA-DR-restricted T lymphocyte responses to HuMBP peptides in patients with MS. To examine which T cell subset proliferates in response to HuMBP peptides, we fractionated the

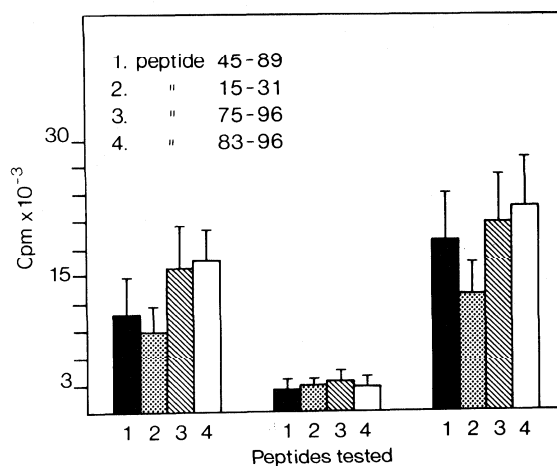


Fig. 5. Response of peripheral blood T lymphocytes (left part) from MS patients to HuMBP peptides following lysis of the T<sub>4</sub> (middle part) or T<sub>8</sub> (right part) subset. Values represent the mean  $\text{cpm} \pm \text{SD}$  from the pooled data (20 MS patients were tested).

MS-derived peripheral T lymphocytes into T<sub>4</sub><sup>+</sup> or T<sub>8</sub><sup>+</sup>-enriched subpopulations by complement depletion of the unwanted population. As shown in Fig. 5 removal of T<sub>4</sub><sup>+</sup>, but not of T<sub>8</sub><sup>+</sup> cells, eliminated the ability to exhibit a proliferative response to HuMBP peptides.

## Discussion

The extensive characterization of MBP has demonstrated the focal nature of certain chemical, encephalitogenic and immunochemical features of the molecule (Carnegie and Moore, 1980; Day, 1982; Whitaker, 1984). The carboxyl half of MBP peptide 45-89 has been found to contain the encephalitogenic determinant for the Lewis rat (Chou et al., 1977) and the epitope recognized in MBP-like material present in human CSF after CNS myelin injury (Whitaker and Seyer, 1979; Whitaker et al., 1980). In a recent report, Price et al. (1986) produced monoclonal antibodies reacting with MBP. This finding demonstrated several immunogenic epitopes within the region of HuMBP 45-89 and in particular of residues 80-89.

In this study we have tested T cell reactivity to synthetic HuMBP peptides 15-31, 75-96, 83-96 and 131-141 as well as to the HuMBP 45-89 peptide fragment. This is the first time HuMBP synthetic peptides have been used to detect primary in vitro proliferative responses from the peripheral blood T lymphocytes of patients with MS. We demonstrated that these peptides could stimulate primary proliferative responses in the peripheral blood T lymphocytes from patients with severe progressive disease. The finding that HuMBP 15-31 synthetic peptide stimulates T lymphocyte proliferation, indicates that other epitopes than those included in the 45-89 region can also be immunogenic. Patients with MS responded almost as well to HuMBP peptides 75-96 and 83-96, indicating that the immunogenic determinants are located within residues 83-96.

Although HuMBP 131-141 did not stimulate T cell proliferation (only two MS patients responded weakly to this peptide) we cannot draw any conclusions for the immunogenicity of the COOH-terminal half of HuMBP. Responses induced by peptide 45-89 were lower than those induced by

synthetic peptides 75–96 and 83–96, localizing the immunogenic epitope to between residues 90–96. The use of more synthetic peptides with overlapping sequences will be necessary to define precisely the T lymphocyte-stimulating epitopes in HuMBP. The responses in the OND group or in the group of normal donors were not significant compared to background responses (i.e. cell cultures in the absence of peptides) with the exception of few cases (19 out of 250, 7.6% of total) which, however, were weak compared with the responses observed in the group with MS.

Recently, Johnson et al. (1986) have demonstrated only slightly higher proliferative responses to HuMBP in patients with active MS compared to those with stable disease, normal control or OND patients. Although the mean responses observed in all four groups were not significantly different (compared to each other), 10% of the patients with active MS responded highly to HuMBP. The authors suggested that at least in some patients there may be an association between sensitization to MBP and disease activity. Although we have not tested MS patients with inactive disease so far, we believe that T cell reactivity to certain MBP epitopes should be in close association with disease activity in many (if not all) patients since all ours proved to be definite responders to HuMBP peptides. An argument against the conclusion reached by Johnson et al. (1986) would be that, during the acute episode of the disease, specific T cells could have been sequestered at the MS plaques thus depleting peripheral blood of the MBP-specific T lymphocytes.

It is very likely that the detection of MBP responders among the MS patients depends on the techniques used. While Johnson et al. (1986) have cultured unseparated peripheral blood lymphocytes with the intact HuMBP, we have used a more precise culture system consisting of pure peripheral blood T lymphocytes and monocytes pulsed with HuMBP peptides. Our system provides better conditions for soluble antigen-induced T lymphocyte proliferation since (a) the responding cell population consists of the highly purified T lymphocytes which represent the proliferating cell type in such systems (Nagy et al., 1981; Klein and Nagy, 1982), (b) the peptide-presenting cells

consist of purified peptide-pulsed monocytes which have been shown to stimulate optimally T lymphocytes in the presence of soluble antigens (Koide et al., 1982; Palacios, 1982; Baxevanis et al., 1988), and (c) the use of synthetic MBP peptides enables the selection of immunogenic epitopes which then elicit higher responses than the intact molecule since, on a molar basis, the immunogenic epitopes on the peptides are in higher concentration in culture than the same ones on the native molecule. For instance, if we had used 100  $\mu\text{g}/\text{ml}$  (final concentration) of intact HuMBP then its molarity in culture (considering a MW of 18 500) would be  $5.4 \times 10^{-6}$  M which in turn is 4–14 times lower than those for the peptides used in this study (Fig. 2). Another advantage in the use of synthetic peptides is that one can also detect segments which lack the presence of immunogenic epitopes (e.g. HuMBP 131–141). Thus, testing of synthetic peptides with overlapping sequence will also help in the determination and analysis of immunogenic epitopes on MBP molecule as well as the responder T cell repertoire.

The T lymphocytes activated upon stimulation with HuMBP peptides possessed the phenotypic markers of inducer/helper cells and were restricted for HLA-DR antigens. The T lymphocytes which proliferate upon stimulation in vitro with antigen have been shown to function as helper cells in antibody production in vitro (Koide et al., 1978; Reinherz et al., 1981; Klein and Nagy, 1982; Morimoto et al., 1985; Baxevanis et al., 1986). Moreover, these T lymphocytes bear the  $T_4^+ 8^-$  phenotype (Meuer et al., 1982; Morimoto et al., 1982; Reinherz and Schlossman, 1983). We have confirmed this observation since we found that  $T_4^+$  but not  $T_8^+$  cells were able to proliferate in response to HuMBP peptides. Thus, it seems likely that the  $T_4^+$  cells which responded to HuMBP peptides in vitro, might represent autoimmune cells in vivo participating in the destruction of myelin in CNS.

It will be important to characterize the regulatory network in which the autoreactive  $T_4^+$  (helper) cell plays a major role and to define the T cell epitopes on the MBP, in order to analyze the diversity of the MBP-specific T cell response. Assay of the proliferative responses of T lymphocytes to HuMBP fragments could be useful as an

additional test in the differentiation of MS from other neurological diseases.

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