Antagonistic Effects of Human Cyclic MBP$_{87-99}$ Altered Peptide Ligands in Experimental Allergic Encephalomyelitis and Human T-Cell Proliferation

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The immunodominant myelin basic protein (MBP) peptide comprising residues 87−99 is a self-antigen in multiple sclerosis (MS). In Lewis rats this epitope induces experimental allergic encephalomyelitis (EAE), a demyelinating disease of the central nervous system, and is a model of MS. Structure−activity studies have shown that Lys^{91} and Pro^{96} residues are important for encephalitogenicity. Replacement of Lys and/or Pro residues with Arg and/or Ala, respectively, results in suppression of EAE. A potent linear altered peptide ligand of the immunodominant sequence MBP$_{83-99}$ has been selected for clinical trial (*Nat. Med.* **2000**, *6*, 1167, 1176). In the present report, two cyclic analogues, cyclo(91−99)[Ala^{96}]MBP$_{87-99}$ and cyclo(87−99)[Arg^{91}, Ala^{96}]MBP$_{87-99}$ were designed by NMR and molecular modeling data on human MBP$_{87-99}$ epitope (Val^{87}-His-Phe-Phe-Lys-Asn-Ile-Val-Thr-Pro-Arg-Thr-Pro^{99}) and its linear antagonist peptide analogue [Arg^{91}, Ala^{96}]MBP$_{87-99}$. These analogues (altered peptide ligands) inhibited EAE in Lewis rats and decreased inflammation in the spinal cord. In addition, the analogue cyclo-(87−99)[Arg^{91}, Ala^{96}]MBP$_{87-99}$ induced proliferation of human peripheral blood T-cells. These cyclic MBP$_{87-99}$ peptide analogues may lead to the design of potent antagonist mimetics for treating MS.

Introduction

Experimental allergic encephalomyelitis (EAE) is an animal model for multiple sclerosis (MS) and is induced in genetically susceptible animals by immunization with self-antigens derived from central nervous system (CNS) myelin. The two principle myelin proteins that have been extensively characterized for induction of EAE are myelin basic protein (MBP) and proteolipid protein (PLP).[1,2] Myelin oligodendrocyte glycoprotein (MOG) has also been used to effectively induce EAE in several animal models. EAE is a CD4+ T-cell-mediated disease,[1,2] and it is characterized by the invasion of the CNS by inflammatory cells, followed by demyelination and paralysis.[2,3] MS in humans is believed to have a similar autoimmune etiopathology, and candidate autoantigens include constituents of the myelin sheath such as MBP and PLP.

Modern approaches toward the therapeutic management of MS involve the design and use of peptide analogues of disease-associated myelin epitopes to induce peripheral T-cell tolerance.[4,5] Altered peptide ligands (APL), which are analogues of immunodominant epitopes such as 87−99, 84−102, 87−106, and 83−99, have been shown to be successful in blocking the development of EAE induced by the parent peptides.[6−9] It has been suggested that such altered ligands cause an antagonistic effect (i.e., loss of T-cell activation) by loss of H-bond contacts between the peptide and T-cell receptor (TCR). In addition APL can switch Th1 immune response toward Th2, thus reversing disease. Furthermore, analogues of MBP or altered peptide ligand can inhibit disease through priming T cells that selectively produce IL-10.[10] Nevertheless, the use of peptides as therapeutic entities is limited because of their sensitivity to proteolytic enzymes. To address the need for a stable molecule with the same biological activity for clinical purposes, it is necessary to use either cyclic peptides that are more resistant to proteolytic hydrolysis or non-peptide mimetics of the parent peptide. The advantages of using cyclic analogues versus linear counterparts are as follows. (i) The conformation of a cyclic analogue is locked compared to the conformational flexibility characterizing the linear counterpart. Cyclization confirms or eliminates suggested active conformations. (ii) A cyclic analogue is a more stable molecule and thus is more resistant to enzymatic degradation, a quality that makes it an attractive candidate as a drug lead. (iii) A cyclic analogue is an important intermediate step toward the rational design and development of a non-peptide drug for oral administration. (iv) Cyclization of amino acid sequences

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results in increased receptor selectivity and bioavailability consequently in a better pharmacological profile.[11] The utility of backbone cyclization has been well appreciated in peptides, and it has been demonstrated for a large number of peptidic systems in which biological activity and in vivo stability can be improved by cyclization.[12] In particular, cyclic peptides have been used in several cases as potent inhibitors of transcriptional activation by Tat protein in human cells,[13] synthetic immunogens,[14] antigens for herpes simplex virus,[15] inhibitors against α-amylase,[16] and protein stabilizers.[11] For diabetes, a cyclic peptide protected mice from disease while the linear counterpart did not.[17,18] Even in its infancy, mimetic strategy is a challenging perspective for medicinal chemists and worth pursuing particularly for MBP epitope-based MS treatment.[19−21]

In this study we developed antagonist cyclic analogues based on the immunodominant human MBP epitope 87−99 (MBP$_{87-99}$). In particular, two cyclic analogues, cyclo(91−99)[Ala^{96}]MBP$_{87-99}$ (**P3**) and cyclo-(87−99)[Arg^{91}, Ala^{96}]MBP$_{87-99}$ (**P4**), have been designed on the basis of conformational studies carried out on MBP epitope 87−99 (**P1**) and its linear antagonist [Arg^{91}, Ala^{96}]MBP$_{87-99}$ (**P2**), which has been recently shown to inhibit EAE induced in Lewis rats by the MBP$_{72-85}$ epitope.[22] These studies have revealed a possible head-to-tail intramolecular proximity for both peptides (nuclear Overhauser effect (NOE) connectivity between γVal^{87}-NHArg^{97}, ϵNHLys^{91}-ω/ω'Arg^{97} for **P1** and αPhe^{89}-δ_2Pro^{97}, αVal^{87}-βThr^{98} for **P2**) among other low-energy linear conformations. On the basis of the possible cyclic conformation of antagonist peptide **P2**, we therefore synthesized the cyclic analogues **P3** and **P4** of peptide **P2** by connecting the side chain amino group of Lys^{91} with the C-terminal carboxyl group and the N-terminus with the C-terminus, respectively. Head-to-tail cyclic analogue **P4** contained residues Arg^{91} and Ala^{96} contributing to inhibitory effects, while the Lys^{91} side chain to the C-terminus cyclic analogue **P3** contained Ala^{96} but was deprived of the Lys^{91} amino group important for agonist activity. Since the human MBP$_{87-99}$ peptide showed weak encephalitogenic activity in Lewis rats, the altered peptide ligands (linear analogue **P2** and cyclic analogues **P3** and **P4**) were screened in EAE induced by the more immunogenic rodent guinea pig MBP$_{72-85}$ peptide. Clinical signs of EAE induced by MBP$_{72-85}$ were completely suppressed by head-to-tail cyclic analogue **P4** and to a lesser degree by the Lys^{91} side chain to the C-terminus of the cyclic **P3** analogue.

Inhibition of linear MBP$_{87-99}$ by cyclic **P4** supports a cyclic conformation assumed by the linear **P1** sequence while approaching the T-cell receptor. Moreover, blockade of MBP$_{72-85}$ induced EAE by the unrelated linear peptide **P2** or its cyclic **P4** analogue could indicate that the mechanism of inhibition is not due to binding competition but rather due to the delivery of a negative signal by the antagonist, which overcomes the agonist response possibly through the activation of antigen-specific regulatory T cells or induction of immunosuppressive cytokines such as IL-10 by T cells.
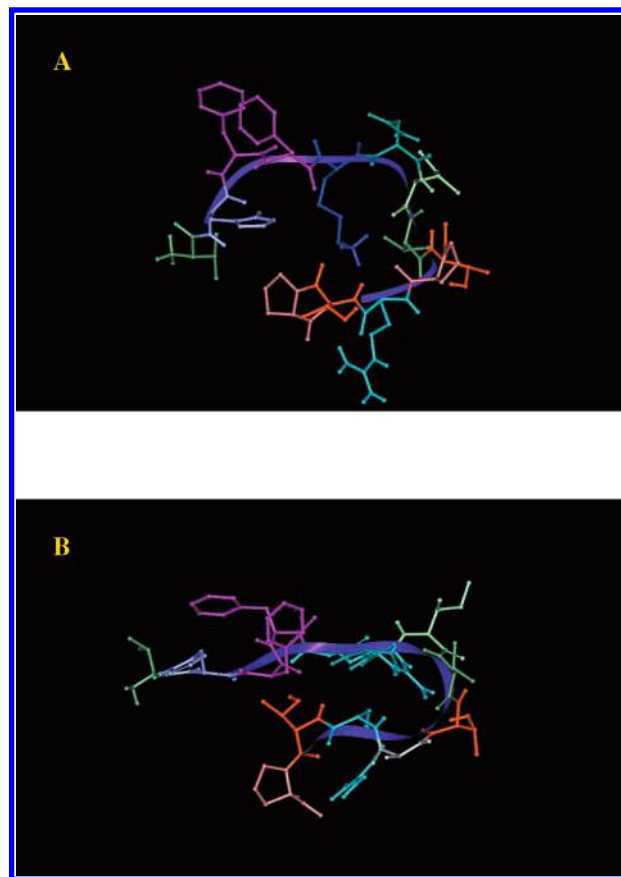

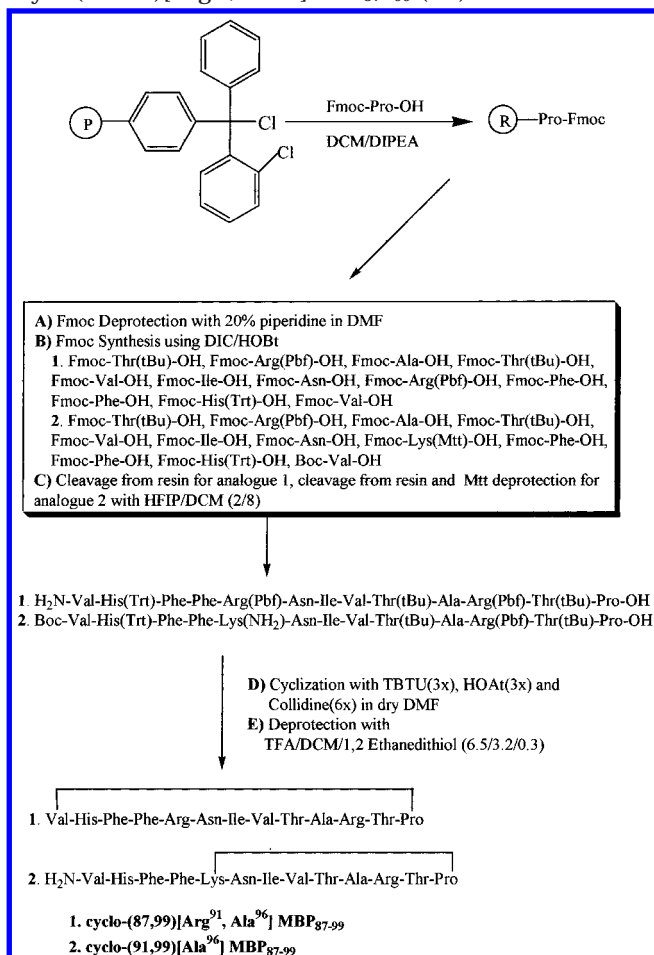**Figure 1.** Low-energy conformer of linear agonist MBP$_{87-99}$ (A) and of linear antagonist [Arg^{91},Ala^{96}]MBP$_{87-99}$ (B).

Results

1. Molecular Modeling and NMR of MBP$_{87-99}$ and [Arg^{91}, Ala^{96}]MBP$_{87-99}$. Linear Agonist (MBP$_{87-99}$). The amino acid spin system was identified by locating networks of characteristic connectivities in the 2D correlation spectra and 2D total correlation spectra. Long-range assignments were made through nuclear Overhauser effect spectrometry (NOESY) experiments using 75 and 150 ms mixing times. The NOE connectivities observed for linear agonist peptide **P1** suggest a possible cyclic conformation for this molecule. A preliminary model based on major NOEs observed in NOESY experiments was built. Specifically, the critical NOEs used as distance constraints to create the model were γVal^{87}-NHArg^{97} and ϵNHLys^{91}-ω/ω'Arg^{97}. These two NOEs suggest a compact molecular structure of the molecule. Lys not only is in proximity to Arg but also is in proximity to the C-terminal carboxylate (COO$^-$) of Pro^{99}. The C-terminal carboxylate of Pro^{99} is also in proximity to the amino terminal of Val^{87}. These electrostatic interactions stabilize the model (Figure 1A). Along with electrostatic interactions, several other hydrogen bonds contribute to its stabilization. The two adjacent phenyl rings (Phe^{89} and Phe^{90}) and imidazole ring of histidine (His^{88}) are not in spatial proximity.

Linear Antagonist ([Arg^{91}, Ala^{96}]MBP$_{87-99}$). As in agonist peptide, amino acid spin system assignment was possible by locating networks of characteristic connectivities in the 2D total correlation spectra. A preliminary model for the antagonist was built based on major NOEs observed in NOESY experiments. Specifically, the critical NOEs used as distance constraints to create the

Scheme 1. Synthetic Procedure for Cyclic Analogues Cyclo(91−99)[Ala^{96}]MBP$_{87-99}$ (**P3**) and Cyclo(87−99)[Arg^{91}, Ala^{96}]MBP$_{87-99}$ (**P4**)

Table 1[a]

peptide	RP-HPLC t_R, min	ESI-MS (M + H$^+$), m/z
cyclo(87−99)[Arg^{91}, Ala^{96}]MBP$_{87-99}$	9.6	1539.7
cyclo(91−99)[Ala^{96}]MBP$_{87-99}$	10.8	1513.2

[a] Conditions of RP-HPLC: column, Nucleosil C18, 250 mm × 4.0 mm; gradient separation, 20% B to 100% B in 27min (B is TFA/AcN 0.08%).
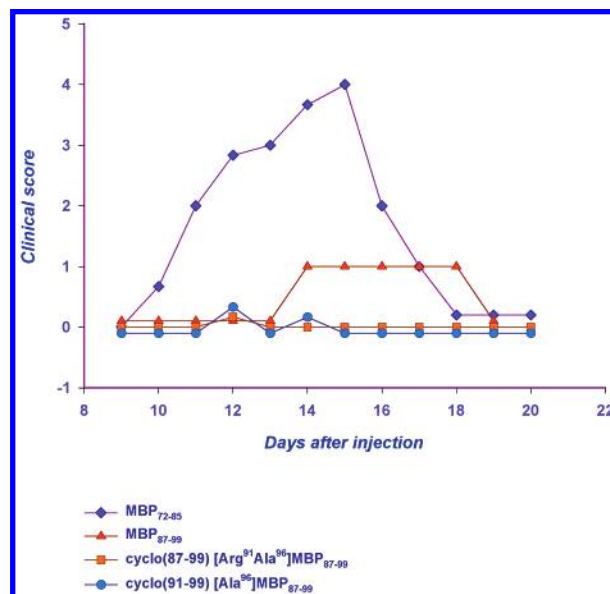

Figure 2. Induction of EAE in Lewis rats. EAE is weakly induced by human MBP$_{87-99}$ but not by its altered ligands cyclo(87−99)[Arg^{91}Ala^{96}]MBP$_{87-99}$ or cyclo(91−99)[Ala^{96}]-MBP$_{87-99}$. Adult female Lewis rats were injected with agonist guinea pig MBP$_{72-85}$, agonist human MBP$_{87-99}$, antagonist (altered peptide ligand) human peptide cyclo(87−99)[Arg^{91}, Ala^{96}]MBP$_{87-99}$, or antagonist human cyclo(91−99)[Ala^{96}]-MBP$_{87-99}$. Acute monophasic EAE was induced only by guinea pig MBP$_{72-85}$ and weakly by human MBP$_{87-99}$.

model were αPhe^{89}-δ$_2$Pro^{97} and αVal^{87}-βThr^{98}, suggesting that the antagonist peptide may adopt a cyclic form. Several but fewer hydrogen bonds are possible in the model (Figure 1B). The substitution of Lys^{91} with Arg and Pro^{96} with Ala has significant effects on the model. For example, Arg^{91}, in contrast to Lys^{91}, does not contribute to any significant interactions with other amino acids.

2. Chemistry. Scheme 1 shows the synthesis of cyclic MBP analogues. In particular, the synthesis of the head-to-tail cyclic peptide antagonist, **P4**, as well as of the Lys^{91} side chain to the C-terminus cyclic analogue **P3**, was carried out by the Fmoc/tBu methodology, utilizing the 2-chlorotrityl chloride (CLTR-Cl) resin that was used previously for the synthesis of novel cyclic amide-linked analogues of MBP$_{72-85}$[23,24] as well as of the thrombin receptor SFLLR motif.[25,26] The side chains of amino acids were protected as follows: Trt for His; Pbf for Arg; tBu for Ser, Thr, Asp, Glu; and Boc for Lys. In the case of **P3** in which Lys^{91} amino and C-terminal carboxyl groups formed an amide bond, we used Mtt in a linear precursor peptide to protect Lys^{91}. This group was removed upon treatment of the protected peptide resin with the splitting mixture dichloromethane (DCM)/ 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) (7/3) used to cleave the peptide from resin. The use of the 2-chloro-trityl resin, as well as of mild cleaving conditions, allowed peptide release from the resin and the subse-

quent cyclization of the desired protected peptide. Cyclization was achieved using *O*-benzotriazol-1-yl-*N,N,N′,N′*-tetramethyluronium tetrafluoroborate (TBTU) and 1-hydroxy-7-azabenzotriazole, 2,4,6-collidine allowing fast reaction and high-yield cyclization product. The cyclization reaction was monitored using the ninhydrin test and analytical HPLC. The reaction mixture was resolved by thin-layer chromatography using a *n*-butanol/acetic acid/water (4/1/1) solvent system. The protected cyclic analogue was then deprotected with 65% trifluoroacetic acid (TFA) + 3% EDT in DCM. The purification of free cyclic products was achieved using high-performance HPLC reversed-phase chromatography. The peptide purity and identification were assessed respectively by analytical RP-HPLC and by mass spectrometry (ESIMS). Table 1 shows the HPLC retention time and MS data of two cyclic MBPs.

3. Inhibition of EAE by Human Cyclo(87−99)-[Arg^{91}, Ala^{96}]MBP$_{87-99}$ and Cyclo(91−96)[Ala^{96}]-MBP$_{87-99}$ Altered Peptide Ligands. As we have previously described,[19,23] guinea pig MBP$_{72-85}$ induces acute monophasic EAE when injected subcutaneously in Lewis rats. The injection of several doses of human MBP$_{87-99}$ peptide also results in the induction of EAE in Lewis rats but with milder clinical signs (Figure 2). In contrast, immunization with the cyclic **P4** or cyclic **P3** did not result in the induction of EAE (Figure
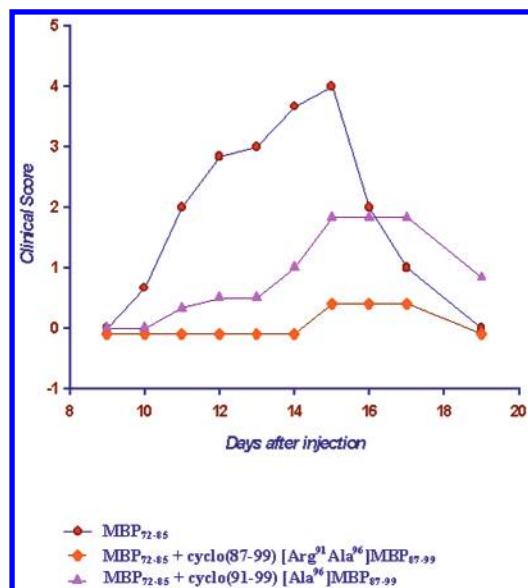
Figure 3. Prevention of EAE in Lewis rats. Adult female Lewis rats were immunized with the agonist guinea pig MBP$_{72-85}$ alone or together with the altered ligands cyclo(87−99)[Arg^{91}, Ala^{96}]MBP$_{87-99}$ or cyclo(91−99)[Ala^{96}]MBP$_{87-99}$ at a ratio of 1:17 agonist/antagonist. Acute monophasic EAE was developed in rats immunized with guinea pig MBP$_{72-85}$ alone, while EAE development was prevented by the coadministration of cyclo(87−99)[Arg^{91}, Ala^{96}]MBP$_{87-99}$. In contrast, coadministration of the altered ligand, cyclo(91−99)[Ala^{96}]MBP$_{87-99}$ resulted in a delay in the onset of the clinical signs.

2). These peptide analogues, when co-injected with guinea pig MBP$_{72-85}$, were found to decrease the development of clinical signs of EAE (Figure 3). Specifically, head-to-tail analogue cyclic **P4** completely blocked the development of EAE induced by guinea pig MBP$_{72-85}$, while the Lys^{91} side chain to the C-terminus analogue cyclic **P3** exerted much weaker antagonistic activity in the development of EAE with delayed onset (Figure 3). Binding factors such as residue 91 (blocked in **P3**), together with conformational changes, may result in a lower antagonist activity of **P3** compared to **P4**. We are currently pursuing the determination of the mechanism of action of the antagonistic effects that MBP$_{87-99}$ has on MBP$_{72-85}$ induced EAE.

4. Histopathological Features. Tissue samples from Lewis rats injected with MBP$_{72-85}$ peptide administered with adjuvant showed perivascular infiltrates of mononuclear cells around small vessels of the spinal cord, particularly between the white and gray matter. Scattered small lymphocytes were also observed in the adjacent parenchyma. In contrast, no inflammation was noted in any of the spinal cord samples taken from the other groups: nonimmunized normal control ($n = 5$), adjuvant alone ($n = 5$), cyclo(87−99)[Arg^{91}, Ala^{96}]MBP$_{87-99}$ ($n = 5$), MBP$_{72-85}$+cyclo(87−99)[Arg^{91}, Ala^{96}]MBP$_{87-99}$ ($n = 5$) (Figure 4). Tissue samples from the liver, the spleen, and mesenteric lymph nodes showed unremarkable changes in all the groups.

5. Effect of Peptides on Human Peripheral Blood T Cells. The immunogenicity of human peptide analogues, linear **P2** and cyclics **P3** and **P4** human peptides, were tested on human adult peripheral blood mononuclear cell (PBMC) cultures. Dose−response curves depicting the numbers of viable cells versus peptide concentration were constructed 72 h later, and the
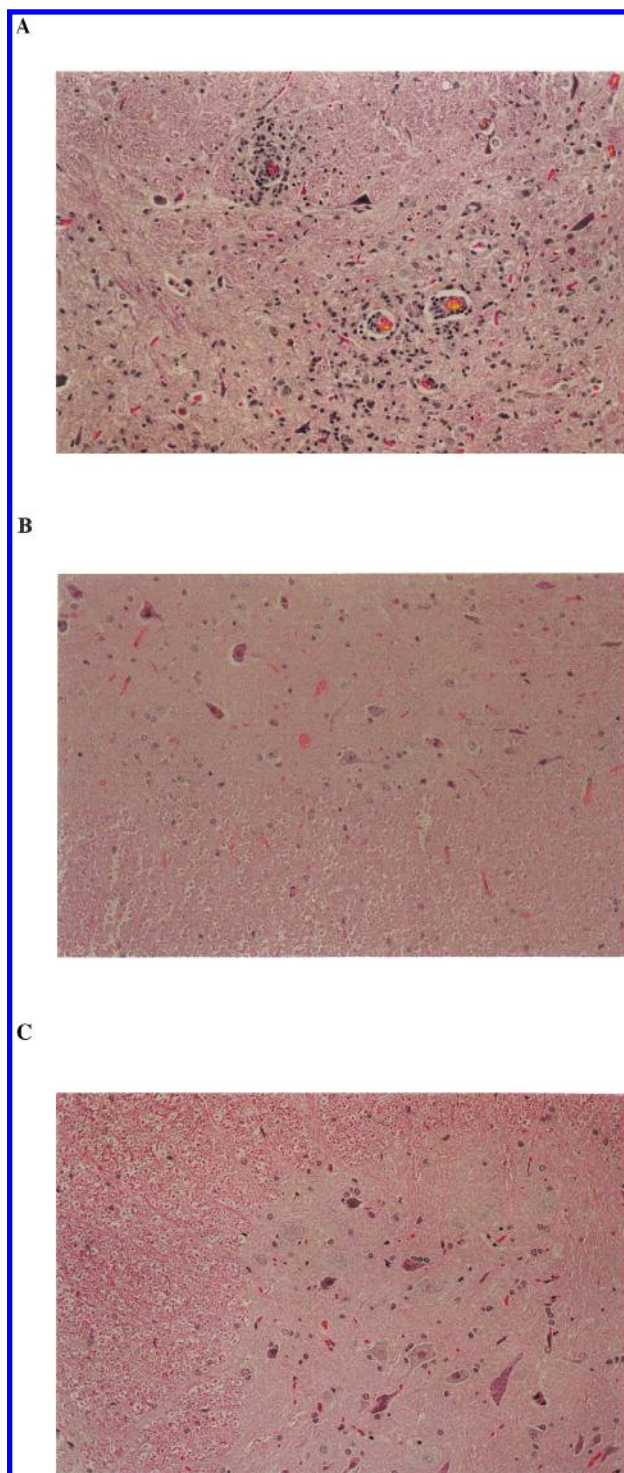

Figure 4. (A) Cross section of the spinal cord from a rat immunized with linear MBP$_{72-85}$ showing full-blown clinical EAE. Perivascular accumulations of inflammatory cells around blood vessels and within the boundaries between the white and gray matter are evident. (B, C) Inflammatory cells are absent in the same anatomical region from a rat immunized with linear MBP$_{72-85}$ + cyclo(87−99)[Arg^{91}, Ala^{96}]MBP$_{87-99}$ (B) and from negative control group (C). (hematoxylin−eosin, ×200)

results are shown in Figure 5. When the human peptide **P2** or **P3** was added to the cells, the number of T cells decreased in a manner independent of the peptide's concentration. Specifically, the addition of the peptide **P2** decreased the T-cell number between 0.690-fold (at a peptide concentration of 1 ng/mL) and 0.642-fold (at
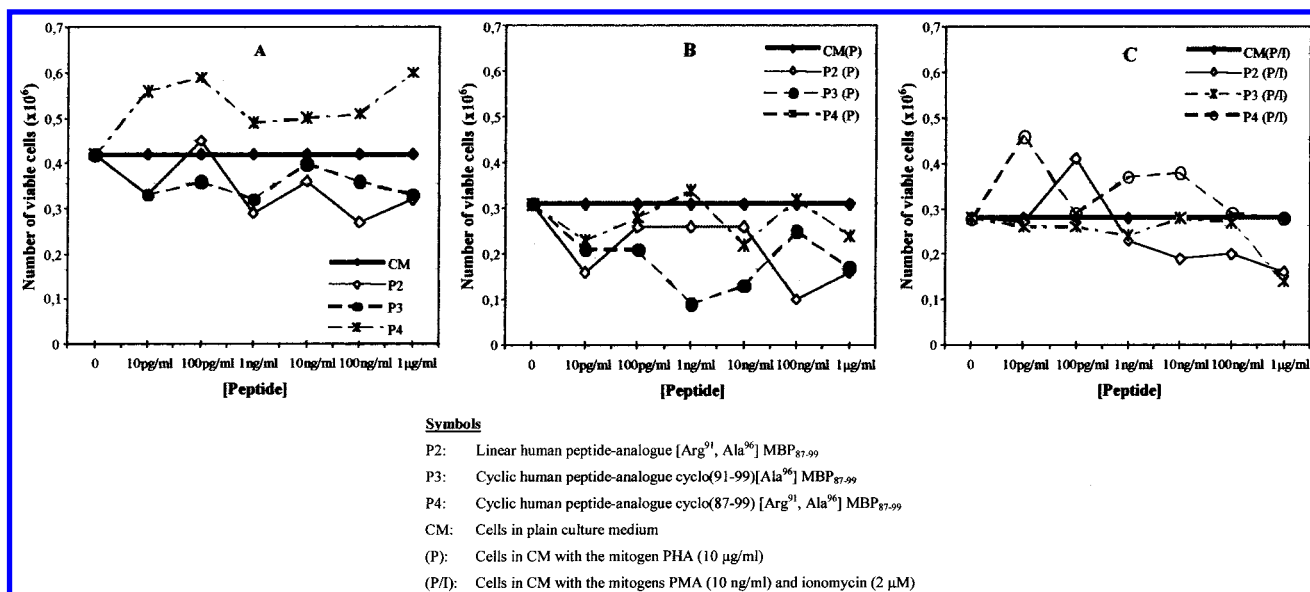
Symbols

P2:   Linear human peptide-analogue [Arg^{91}, Ala^{96}] MBP$_{87-99}$

P3:   Cyclic human peptide-analogue cyclo(91-99)[Ala^{96}] MBP$_{87-99}$

P4:   Cyclic human peptide-analogue cyclo(87-99) [Arg^{91}, Ala^{96}] MBP$_{87-99}$

CM: Cells in plain culture medium

(P):   Cells in CM with the mitogen PHA (10 μg/ml)

(P/I):   Cells in CM with the mitogens PMA (10 ng/ml) and ionomycin (2 μM)

Figure 5. Effect of human peptides on cell numbers of peripheral blood mononuclear cells (PBMC) from normal donors. (A) Cells were cultured for 72 h in the presence of different peptide concentrations, and viable cells were counted by a hemocytometer. The *X* axis represents the concentrations of the peptide analogues tested, and the *Y* axis represents the number of viable cell ($\times 10^6$). (B) Cells were cultured for 72 h in the presence of different peptide concentrations and the mitogen PHA (10 μg/mL). (C) Cells were cultured for 72 h in the presence of different peptide concentrations and the mitogens PMA (10 ng/mL) and ionomycin (2 μM).

a peptide concentration of 100 ng/mL). The addition of the peptide **P3** decreased the T-cell number about 0.785-fold (at a peptide concentration of 10 pg/mL), 0.760-fold (at a peptide concentration of 1 ng/mL), and about 0.785-fold (at a peptide concentration of 1 μg/mL) (Figure 5A). In contrast, the addition of the cyclic human peptide analogue **P4** caused an overall increase in T-cell numbers reaching a maximum (1.428-fold) at a peptide concentration of 1 μg/mL. The difference among the peptides **P2**, **P3**, and **P4** is that peptide **P4** represents the cyclic form of a doubly mutated epitope of human MBP$_{87-99}$, which was derived by substitution of the amino acids Lys^{91} and Pro^{96} by Arg^{91} and Ala^{96} and full-length cyclization. It is possible that the full-length cyclization and/or the double substitution of the amino acids Lys^{91} and Pro^{96} by Arg^{91} and Ala^{96} in peptide **P4** created a peptide that ceased to be recognized as "self" by normal T cells and, hence, caused a weak antigenic-type reaction by the T cells (Figure 5A).

To test whether these peptides can act as accessory-type molecules for T cells, PBMC from healthy donors were cultured in the presence of the mitogens PHA or PMA/ionomycin. The presence of PHA in the cell cultures resulted in a peptide-dependent decrease in cellular proliferation (Figure 5B). The addition of peptide **P2** decreased T-cell proliferation rates between 0.516-fold and 0.322-fold at peptide concentrations of 10 pg/mL and 100 ng/mL, respectively. A similar pattern was observed with **P3**, where the proliferation rates fell by 0.290-fold (1 ng/mL) to 0.419-fold (10 ng/mL). The only peptide that had a slightly positive effect on the proliferation rate of T-cells in the presence of mitogen PHA was **P4** (1.096-fold at a concentration of 1 μg/mL). When the cells were cultured in the presence of the mitogens PMA and ionomycine (Figure 5C), the proliferation curves showed an increase only for peptides **P2** and **P4**. Peptide **P2** increased T-cell number with a maximum of about 1.464-fold (at a peptide concentration

of 100 pg/mL), and peptide **P4** caused an increase between 1.642-fold and 1.357-fold at peptide concentrations of 10 pg/mL and 100 ng/mL, respectively. Nevertheless, since the combination of PMA and ionomycin is a strong mitogenic response of T cells, it is not clear whether the slight variations in proliferation rates, observed in the presence of the peptides, have a biological significanse. It remains to be seen what the effect of the peptides will be in T-cell cultures from MS patients.

Discussion

The potential importance of MBP$_{83-99}$ in human studies of MS stems from a number of findings.[7,8] MBP peptides (89−101) and (87−99) are encephalitogenic in rodent strains susceptible to acute (Lewis rat) and chronic (SJL mouse) EAE.[27] Because MBP is one of the candidate autoantigens in MS and the MBP$_{87-99}$ epitope represents the most immunodominant region in human MS,[28,29] the design of analogues with immunomodulatory activity is of great importance. There is gathering evidence that mutations of disease-associated epitopes (altered peptide ligands) can actively inhibit disease through the loss of H-bond contacts between the peptide−MHC and TCR as well as the induction of antigen-specific suppressor T cells. In addition, altered peptide ligands (agonist to antagonist) can prime T cells that selectively produce IL-10, thus switching immune responses from IFN-γ to IL-10. Therefore, peptide analogues based on immunodominant epitopes of MBP could be used to prevent and treat disease.[30]

Development of novel alternative molecules that mimic the immunomodulatory activity of MBP epitope peptides and maintain an advantage over regular peptides in terms of stability is a necessary step before these molecules can be used for therapeutic purposes. There are several approaches in the development of such molecules. One is the design and synthesis of non-

peptide mimetics with the same biological activity as the parent peptide or with antagonist activity. Another approach is the design of constrained cyclic analogues. Cyclic analogues of MBP$_{87-99}$ offer several advantages compared to its linear counterparts, such as increased resistance to metabolic degradation and conformational restriction. A potent constrained peptide analogue offers important structural information regarding the bioactive conformation and the pharmacophoric groups for drug discovery and development. In this regard appropriate cyclization of human MBP$_{87-99}$ epitope altered peptide analogues may reduce antigenicity as shown by linear altered peptide ligand in recent clinical phase I and II trials.[7,8]

Human MBP$_{87-99}$ peptide induces EAE; however, the replacement of either Lys with Arg at position 91 or Pro with Ala at position 96 results in analogues with inhibitory effects.[6,10] In this work, both residues Lys^{91} and Pro^{96} of human MBP$_{87-99}$ were replaced with Arg and Ala, resulting in a potent antagonist peptide in the MBP$_{72-85}$ EAE model. Furthermore, the cyclic analogues cyclo(87−99)[Arg^{91},Ala^{96}]MBP$_{87-99}$ and cyclo(91−99)-[Ala^{96}]MBP$_{87-99}$ were synthesized by amide bridging the N- and C-terminal residues 87, 99 and Lys side chain and C-terminal residues 91, 99, respectively, of the linear antagonist [Arg^{91}, Ala^{96}]MBP$_{87-99}$. The cyclic analogues were designed on the basis of the conformational properties of the agonist and antagonist linear analogues, which may adopt cyclic conformations among other low-energy conformers as revealed by the NMR studies. Indeed, the NOESY data for both compounds indicated proximity of the N- and C-terminal domains. The molecular dynamics approach, in which the structure was manipulated to fulfill the observed NOEs between γVal^{87}-NHArg^{97} and ζNHLys^{91}-nNHArg^{97} in agonist peptide **P1**, yielded a cyclic conformational model (Figure 1A). Similarly, two important NOEs (αPhe^{89}-δ_2Pro^{97}, αVal^{87}-βThr^{98}) observed in peptide **P2** together with other major observed NOEs were used to develop a conformational model for the antagonist peptide characterized also by a pseudocyclic conformation (Figure 1B). Thus, two cyclic analogues were synthesized to test activity and the conformational model of the linear agonist and linear antagonist peptides. Activities justified NMR-based conformational modeling.

Because human MBP$_{87-99}$ is a weak effector of EAE in Lewis rats (Figure 2), guinea pig MBP$_{72-85}$ was used as the disease-producing antigen in these studies. The linear peptide **P2**, which is an antagonist analogue of MBP$_{87-99}$, was recently reported to prevent EAE when co-injected with MBP$_{72-85}$.[22] Cyclization of the linear antagonist at appropriate positions resulted in two cyclic peptides **P3** and **P4**, which also prevented, in different degrees, EAE induced by MBP$_{72-85}$ (Figure 3).

Lower antagonist activity of the Lys^{91} side chain to the C-terminus analogue **P3** compared to head-to-tail analogue **P4** may be attributed to lower binding ability of **P3** because critical residue 91 is blocked by the amide bond formation with subsequent conformational changes affecting activity. Furthermore, lack of agonist activity in the EAE system and expression of antagonist activity by the head-to-tail cyclic analogue **P4** suggests that terminal charged groups are not required for recognition

by TCR and that critical residues for conformation and potency are amino acids Lys and Pro at positions 91 and 96.

The finding that EAE induced by guinea pig MBP$_{72-85}$ can be reversed by an analogue of a human epitope MBP$_{87-99}$ is intriguing. Simplistic explanations, such as direct competition at TCR sites, are not compelling because of the gross dissimilarities in the structural and physicochemical properties of the two epitopes and their analogues, as elaborated in detail previously.[22] Competition at MHC sites are apparently less discriminating than TCR and may bind a variety of peptide structures. This does not provide a viable explanation because there is no reason to suppose that the number of available MHC sites would be limiting, so both peptides would be expected to be bound to MHC molecules. On the other hand, one potentially more reasonable explanation might be derived from "epitope spreading", a well-known phenomenon in which the T-cell response to one epitope of a protein changes with time to response to other epitopes of the protein (a factor that has made it difficult to identify causal, as opposed to dominant, epitopes in autoimmune diseases). Thus, it is conceivable that early immunogenicity toward MBP$_{72-85}$ eventually spreads to include recognition sites on MBP$_{87-99}$ such that the dominant immune response evolves antibodies that recognize structural elements in spanning residues of 72−85 sequence of guinea pig MBP. Accordingly, antagonist analogues of human MBP$_{87-99}$ would be able to prevent binding of antibodies to the combined elements of MBP$_{72-85}$ and MBP$_{87-99}$. Other more complex explanations, involving cross talk between different populations of T cells, and the possible recruitment of suppressor T cells that obviate the EAE response should also be considered.[22]

Furthermore to the above explanations, altered peptide ligands can switch an agonist peptide into an antagonist peptide. Peptides that are involved in the trimolecular complex (MHC−peptide−TCR) and that cause an antagonistic effect (i.e., loss of T-cell activation) have loss of H-bond contacts of peptide side chains with the CDR3 loops of the TCR.[31] Mutation of a large side chain of the peptide that interacts with the TCR to small side chain amino acids (such as Ala, Gly, Ser) can cause antagonism and thus inhibits disease. The crystal structure of MBP$_{87-99}$ and [Arg^{91}, Ala^{96}]MBP$_{87-99}$ would be important for elucidating the interactions between peptide−MHC and TCR. Furthermore, altered peptide ligands can elicit selected effects or functions from T cells.[32] In malaria and in autoimmune diseases, altered peptide ligands can be used to switch an epitope-specific Th1-type CD4+ T-cell response (characterized by production of IFN-γ, IL-2) toward a Th2-type T cell response (IL-4, IL-10, TGFβ), resulting in disease suppression. The recruitment of distinct populations of suppressor T cells to the autoimmune target, by activation through different TCRs, is also possible. Such cells could then turn off the Th1-type T cells through potent immunosuppressive feedback loops involving IL-10, again resulting in disease suppression. In this study, it is likely that the antagonistic MBP$_{87-99}$ peptides activate a distinct population of MBP-specific T cells compared to those induced by the encephalitogenic MBP$_{72-85}$ peptide and that these cells have the capacity

to potently neutralize EAE. It will be interesting to determine the mechanism by which such cells act and, importantly, whether this involves the action of IL-10, an important therapeutic goal of mimetic studies. These studies in mice could also be directly applicable to human studies. Findings of human cyclo(87−99)[Arg[91], Ala[96]]MBP$_{87-99}$ on human peripheral lymphocytes indicate that this peptide weakly activates T cells probably because these cells recognize this peptide as nonself. Alternatively, the proliferating T cells could be Th2 rather than Th1 clones. We are currently measuring Th1 (IFN-γ, IL-2) and Th2 (IL-4, IL-10) cytokines in these human cell cultures to determine whether MBP$_{87-99}$ induces a Th1 response that may be reversed to a Th2 response by the antagonistic peptide [Arg[91], Ala[96]]MBP$_{87-99}$. It is crucial to repeat these experiments using T cells from MS patients in order to determine whether the cyclo(87−99)[Arg[91], Ala[96]]MBP$_{87-99}$ peptide can be an effective antagonist.

Conclusion

This research has shed light on the conformation properties of the human encephalitogenic MBP$_{87-99}$ sequence, which would allow the design of EAE-suppressing cyclic analogues with improved pharmacological profile in terms of stability, duration of action, and receptor selectivity. Reported studies have shown the importance of Lys and Pro at positions 91 and 96, respectively, for potency, while NMR and theoretical calculations indicate a head-to-tail spatial proximity, suggesting possible cyclic conformations for the linear agonist and antagonist sequences. Utilizing the important feature of N- to C-terminal cyclization and the presence of Lys[91] for antagonist activity in a linear peptide, potent cyclic antagonist peptides were designed and synthesized. The antagonist potency of cyclic peptides provided important conformation and structural information for antagonist activity and will initiate future SAR studies that will further explore positions 91 and 96. If the mechanisms of EAE and MS are similar, cyclic peptides based on MBP$_{87-99}$ may turn out to be viable therapeutic approaches for the treatment of multiple sclerosis.

Experimental Procedures

Solid-Phase Peptide Synthesis of Linear Precyclic Analogues Val-His(Trt)-Phe-Phe-Lys(Boc)-Asn-Ile-Val-Thr(tBu)-Pro-Arg(Pbf)-Thr(tBu)-Pro [I] and Boc-Val-His(Trt)-Phe-Phe-Lys-Asn-Ile-Val-Thr(tBu)-Ala-Arg(Pbf)-Thr(tBu)-Pro [II]. The linear protected peptides **I** and **II** were prepared on 2-chlorotrityl chloride resin (CLTR-Cl) using Fmoc/tBu methodology.[23,25,33,34] The first N^{α}-Fmoc (9-fluorenylmethyloxycarboxyl) protected amino acid [Fmoc-Pro-OH (4.5 mmol, 1.25 g)] was esterified on the resin (3 g, 1.5 mmol of Cl^{-}/g of resin) in the presence of diisopropylethylamine (DIPEA) (13.5 mmol, 2.25 mL) in DCM using the Barlos method.[33] The substitution was found to be 0.65 mmol of amino acid/g of resin. First, the tripeptide H-Arg(Pbf)-Thr(tBu)-Pro-CLTR (4.15 g) was synthesized using Fmoc-Thr(tBu)-OH (4.9 mmol), Fmoc-Arg(Pbf)-OH (4.9 mmol), and Fmoc-Pro-CLTR. After deprotection of Fmoc, coupling was carried out in the presence of N,N'-diisopropylcarbodiimide (DIC) (5.4 mmol) and 1-hydroxybenzotriazol (HOBt) (7.3 mmol) in DMF. In each case the Fmoc protecting group was removed by treatment with piperidine (20% in DMF, 2 × 15 min). The Kaiser test and thin-layer chromatography (TLC) in *n*-butanol/acetic acid/water (4:1:1) (BAW) as elutant verified the completeness of

each coupling or Fmoc deprotection. The synthesized tripeptide was used as a starting material to synthesize the protected peptides **I** and **II** in which the fourth residue is Pro and Ala, respectively. The protected amino acids used were Fmoc-Ala-OH (for **I**); Fmoc-Pro-OH (for **I**); Fmoc-Thr(tBu)-OH, Fmoc-Val-OH, Fmoc-Ile-OH, Fmoc-Asn-OH, and Fmoc-Arg(Pbf)-OH (for **I**); Fmoc-Lys(Mtt)-OH (for **II**), Fmoc-Phe-OH, Fmoc-His-(Trt)-OH, and Fmoc-Val-OH (for **I**); and Boc-Val-OH (for **II**). The protected peptide resin was treated with the splitting mixture DCM/HFIP (7/3, 15 mL) for 3 h at room temperature to remove the peptide from the resin with simultaneous Mtt deprotection from Lys[91] in the case of peptide **II**. The mixture was filtered off, and the resin was washed with the splitting mixture (×2) and DCM (×3). The solvent was removed on a rotary evaporator, and the obtained oily product was precipitated from cold and dry diethyl ether as a white solid. The final crude peptides **I** and **II** were 0.5 g and 0.55 g, respectively (Scheme 1), starting from 2.075 g of tripeptide H-Arg(Pbf)-Thr(tBu)-Pro-CLTR.

Cyclization Procedure of Protected Linear Peptides I and II. To a solution of the above linear protected peptide **I** or **II** (200 mg, 0.09 mmol) in dry DMF (15 mL), 2,4,6-collidine (0.072 mL, 0.54 mmol) and 1-hydroxy-7-azabenzotriazol (36.8 mg, 0.27 mmol) were added. The solution was then added dropwise to a solution of *O*-benzotriazol-1-yl-*N,N,N',N'*-tetramethyluronium tetrafluoroborate (TBTU) (86.7 mg, 0.27 mmol) in dry dimethylformamide (80 mL) for 2 h, and the mixture was stirred for 4 h. The reaction was followed by the ninhydrin test on TLC (BAW). The solvent was removed from the reaction mixture under reduced pressure, affording a light-yellow oily residue. The cyclic protected peptide was precipitated from H$_2$O and was dried in vacuo for 12 h[23,25,26] (Scheme 1).

Preparation of Final Cyclic Analogues. Each protected cyclic peptide was treated with the deprotection mixture DCM/TFA/ethanedithiol/anisole (32/65/2/1) for 4 h at room temperature. The resulting solution was concentrated under vacuum to a small volume (0.5 mL). The final free cyclic peptides were precipitated as a light-yellow amorphous solid by the addition of diethyl ether, filtered, and then were dried in vacuo for 12 h (purity >80%). The final crude products were further purified using RP-HPLC.

HPLC/TLC/ESIMS. Preparative HPLC for cyclic analogues was performed with a Waters system equipped with a 600 controller system using a Lichrosorb RP-18 reversed-phase semipreparative column (250 mm × 10 mm) with 7 μm packing material. Separations were achieved with a stepped linear gradient of acetonitrile (AcN) (0.08%TFA) in water (0.08% TFA) over 50 min at a flow rate of 3 mL/min. Peptide purity was assessed by analytical RP-HPLC, thin-layer chromatography (TLC) (BAW, 4:1:1), and mass spectrometry (ESI-MS).[23,25]

NMR Spectroscopy. NMR-based conformational studies have been based on methods previously described.[35−41] NMR spectra in this research were recorded on a Varian INOVA 600 MHz spectometer, in DMSO-d_6 at 298 K. The sample concentrations were 5 mg/0.4 mL. The double-quantum-filtered correlation spectroscopy (DQF-COSY)[42] and ^{1}H–^{13}C heteronuclear single quantum coherence (HSQC)[43,44] experiments were performed with gradients. The total correlation spectroscopy (TOCSY)[45,46] and NOESY[47] experiments were recorded using standard pulse sequences in the phase-sensitive mode. The TOCSY data were recorded with an MLEV-17[5] mixing sequence of 60 ms and a 10 kHz spin-lock field strength. NOESY data were obtained at mixing times of 75 and 150 ms. The ^{1}H sweep width was 11 400 Hz. Typically, the homonuclear proton spectra were acquired with 4096 data points in t_2, 16−32 scans, 324−412 complex points in t_1, and a relaxation delay of 1−1.5 s. The ^{1}H–^{13}C HSQC spectra were recorded with 1024 data points in t_2, 32 scans per increment, 128 complex points in t_1, and a relaxation delay of 1 s. The ^{13}C spectral width was 25 000 Hz. Spectra were zero-filled two times and apodized with a squared sine bell function shifted by $\pi/2$ in both dimensions.

Molecular Modeling. Computer calculations were performed on a Silicon Graphics O2 using Quanta software package. Molecular mechanics calculations were carried out using the Charmm force field. The peptide structures were first minimized using the minimization algorithms embedded in the package using an energy gradient tolerance of 0.01 kcal mol^{-1} Å. Distance restraints with a combination of dynamics experiments revealed the low-energy conformers and clusters. Details of the strategy used are reported elsewhere.[37]

Induction or Suppression of EAE by Linear or Cyclic Analogues. Female Lewis rats (220 g) were immunized subcutaneously in the hind footpads with guinea pig MBP$_{72-85}$ (30 μg), human MBP$_{87-99}$ (30 μg), cyclo(87−99)[Arg^{91}, Ala^{96}]-MBP$_{87-99}$ (500 μg), or cyclo(91−99)[Ala^{96}]MBP$_{87-99}$ (500 μg) (n = 5 per group). Furthermore, [Arg^{91}, Ala^{96}]MBP$_{87-99}$ (500 μg), cyclic [Arg^{91}, Ala^{96}]MBP$_{87-99}$ (500 μg), and cyclic [Ala^{96}]MBP$_{87-99}$ (500 μg) were co-injected with MBP$_{72-85}$ (30μg) in Lewis rats (n = 5 per group) to test for antagonist effects. For injection, peptides were dissolved in PBS and emulsified in an equal volume of Freund's complete adjuvant (CFA, Difco) containing 400 μg of heat-killed *Mycobacterium tuberculosis* H37Ra (Difco). Clinical EAE was graded on a scale of 0−4 by established criteria as follows: 0, no discernible disease; 0.5, weight loss; 1, flaccid tail; 2, hind limb weakness; 3, paraplegia; 4, paraplegia with forelimb weakness, moribund.

Cell Cultivation. Heparinized venous blood was collected from three randomly selected healthy adult donors (Blood Transfusion Center, Patras University Hospital). Mononuclear cells were prepared by centrifugation over a Ficoll−Paque gradient (Pharmacia, Sweden). The cells were cultured in RPMI1640 culture medium (CM, GIBCO BRL, Gaithersburg, MD) containing 10% fetal calf serum (FCS) (GIBCO BRL, Gaithersburg, MD) and other supplements as described,[48] in the presence or absence of 10 μg/mL per10^6 cells of the mitogen phytohaemaglutinine (PHA) (Sigma, St. Louis, MO) or with a combination of the mitogens phorbol myristate acetate (PMA, 10 ng/mL per 10^6 cells) and ionomycin (1μM) for 72h. When indicated, the cells were cultured with varying doses of the linear peptide [Arg^{91}, Ala^{96}]MBP$_{87-99}$ (agonist), cyclo(87−99)-[Arg^{91}, Ala^{96}]MBP$_{87-99}$ (antagonist), and cyclo(91−99)[Ala^{96}]-MBP$_{87-99}$. At the end of the 72 h culture period, the cells were counted using a hemocytometer, and their numbers were corrected for viability, which was estimated by the trypan-blue exclusion method.[48]

Histological Examination. Immunized rats were sacrificed at the peak of clinical disease (day 15). Spinal cord, liver, spleen, and mesenteric lymph nodes were isolated and fixed in 4% buffered paraformaldehyde overnight at 4 °C. In the case of the spinal cord, the whole cervical and thoracic vertebral column containing the intact spinal cord was removed and immersion was fixed as described above. After fixation, the whole spinal cord was dissected and serially sectioned at about 2 mm intervals and embedded in paraffin. Representative sections were also taken from the liver, the spleen, and mesenteric lymph nodes and were embedded in paraffin. Paraffin sections, 4 μm thick, were stained with hematoxylin and eosin.

Supporting Information Available: A 600 MHz ^{1}H NMR spectrum of cyclo(91−99)[Ala^{96}]MBP$_{87-99}$, analytical RP-HPLC data, ESI-MS spectrum for cyclo(87−99)[Arg^{91}, Ala^{96}]MBP$_{87-99}$ and cyclo(91−99)[Ala^{96}]MBP$_{87-99}$, and selected regions of the 2D 600 MHz ^{1}H−^{1}H NOESY spectra for linear MBP$_{87-99}$ and for linear [Arg^{91} Ala^{96}]MBP$_{87-99}$. This material is available free of charge via the Internet at http://pubs.acs.org.

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