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Treatment of Experimental Allergic Encephalomyelitis (EAE) Induced by Guinea Pig Myelin Basic Protein Epitope 72–85 with a Human $MBP_{87–99}$ Analogue and Effects of Cyclic Peptides

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Abstract—Experimental autoimmune encephalomyelitis (EAE) is an inflammatory and demyelinating disease of the central nervous system and is an animal model of multiple sclerosis (MS). In the present report, a linear analogue and a series of cyclic semi-mimetic peptides were designed and synthesized based on the human myelin basic protein ($MBP_{87–99}$) epitope (Val^{87}-His-Phe-Phe-Lys-Asn-Ile-Val-Thr-Pro-Arg-Thr-Pro^{99}) and on Copolymer I (a mixture of random polymers of Ala, Gln, Lys and Tyr used to treat MS). These analogues were designed looking for suppressors of EAE induced by guinea pig $MBP_{72–85}$ epitope (Gln-Lys-Ser-Gln-Arg-Ser-Gln-Asp-Glu-Asn-Pro-Val) in Lewis rats. The linear analogue [Arg^{91},Ala^{96}]$MBP_{87–99}$, in which Arg substitutes Lys^{91} and Ala substitutes Pro^{96}, was found to be a strong inhibitor which when administered to Lewis rats together with the encephalitogenic agonist $MBP_{72–85}$ completely prevented the induction of EAE. In contrast, three N- and C-termini amide-linked cyclic semi-mimetic peptides, [cyclo-Phe-Arg-Asn-Ile-Val-Thr-Ala-Acp (**1**), cyclo-Phe-Ala-Arg-Gln-Acp (**2**), cyclo-Tyr-Ala-Lys-Gln-Acp (**3**)] as well as a Lys side chain and C-terminous cyclic semi mimetic peptide cyclo(Lys, Acp)-Phe-Lys-Asn-Ile-Val-Thr-Ala-Acp (**4**) which contain segments of $MBP_{87–99}$ or are constituted from immunophoric residues of copolymer I, were ineffective in inducing or inhibiting EAE in Lewis rats. However co-injection of cyclic analogues with $MBP_{72–85}$ delayed the onset of EAE indicating a modulatory effect on the EAE activity of $MBP_{72–85}$. These findings suggest that molecule length, size of cyclic moiety and backbone conformation are important elements for immunogenic activity. Moreover blockade of $MBP_{72–85}$ induced EAE by the unrelated peptide [Arg^{91},Ala^{96}]$MBP_{87–99}$ 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.

Introduction

Multiple sclerosis (MS) is a chronic inflammatory disease of the CNS characterized by focal T cell and macrophage infiltrates, demyelination and loss of neurologic function.[1,2] MS is generally considered to be an autoimmune disease caused by neuroantigen-specific CD4+ T cells. Candidate autoantigens include constituents of the myelin sheath such as myelin basic protein (MBP) and proteolipid protein (PLP), and modern approaches towards the therapeutic management of MS involve the design and use of peptide analogues of disease-associated myelin epitopes to induce peripheral T cell tolerance.[3,4] MBP is hypothesized to be a potential autoimmunogen in MS, largely because injection with MBP, or the administration of activated MBP-reactive T cells induces experimental autoimmune encephalomyelitis (EAE) in animal models. EAE is one of the best studied experimental animal models of MS, and represents an invaluable in vivo system for the evaluation of therapeutic approaches. EAE is a CD4+ T cell-mediated disease[5] that can be induced by immunization with MBP or PLP proteins or peptide epitopes. In Lewis rats, encephalitogenic T cells recognising the 72–85 amino acid sequence of guinea pig MBP ($MBP_{72–85}$) dominate the immune response.[6–8] The suggestion has been made that disease can be modulated with peptides that interfere

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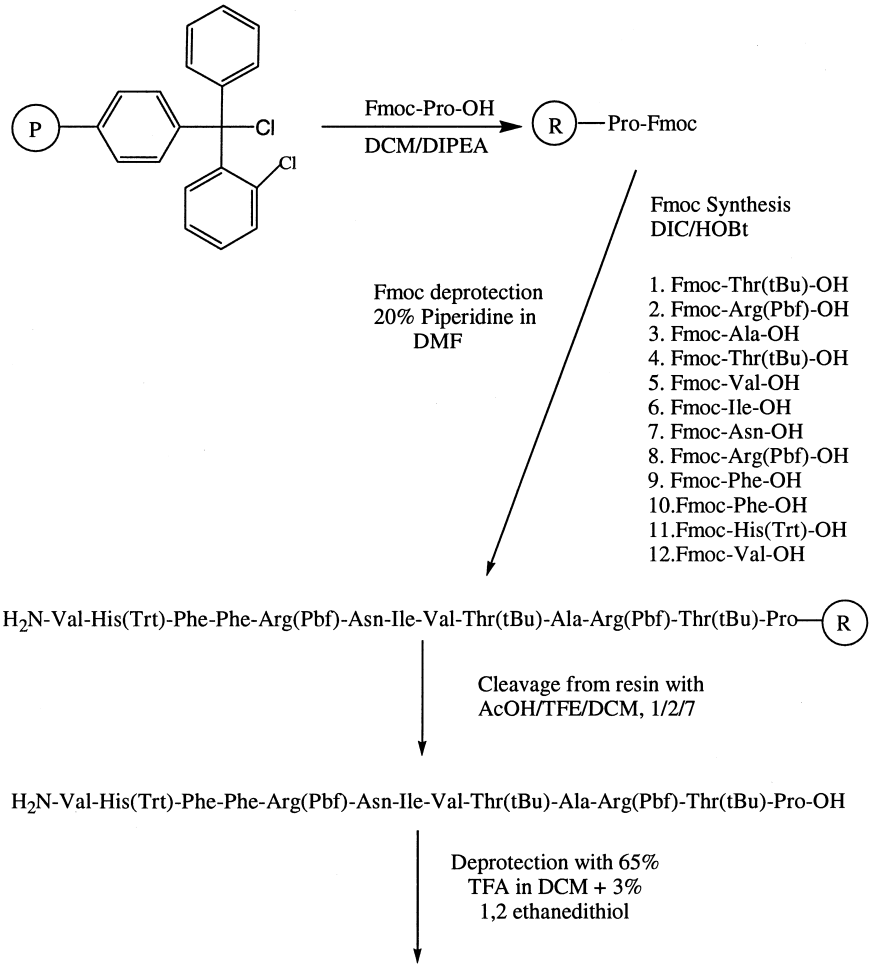
with the formation of the trimolecular complex MHC-peptide-T cell receptor, although there is gathering evidence that analogues of MBP can inhibit disease through the activation of antigen-specific regulatory T cells.[9,10]

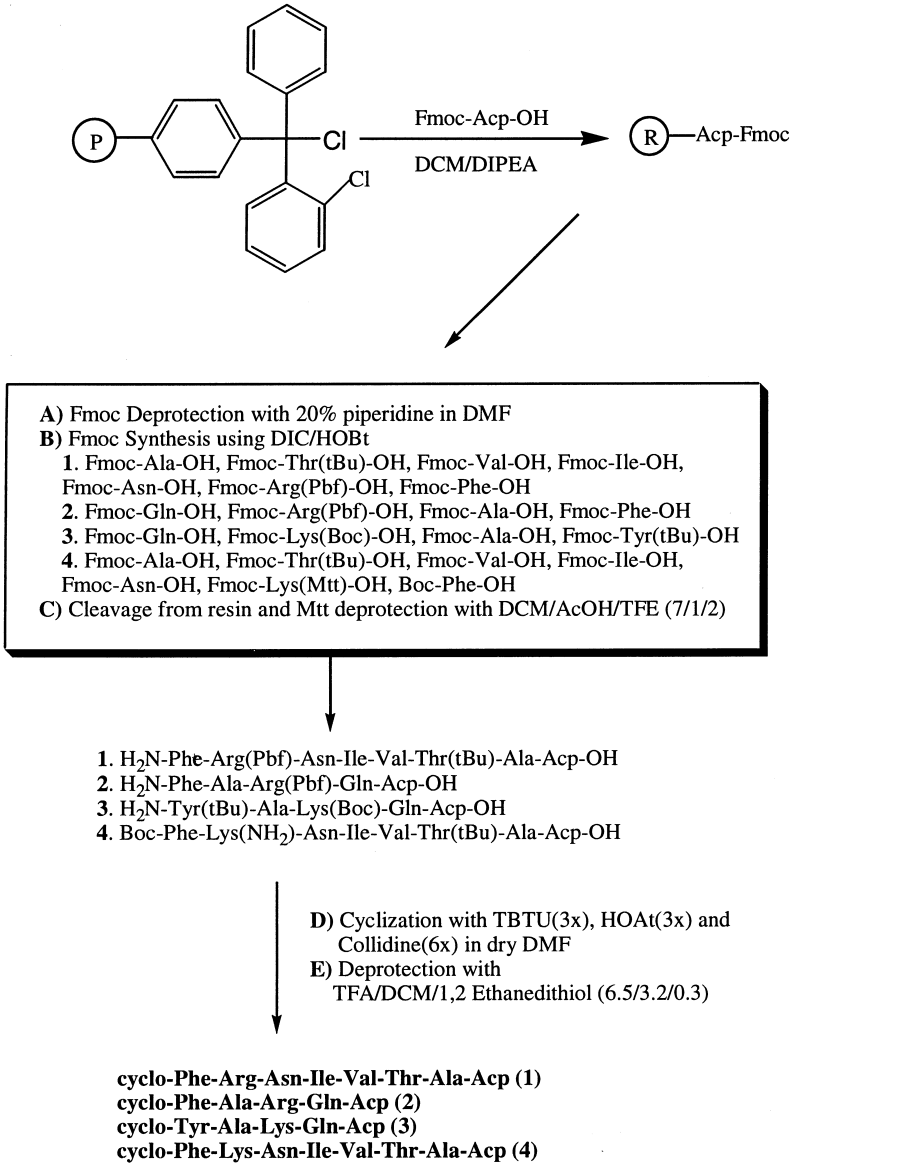
In this work, we pursued the development of an antagonist peptide based on human MBP epitope 87–99, that has been shown to induce EAE in rodents. We also pursued the development of cyclic analogues with possible inhibitory or immunomodulatory effects, which might interfere in the formation of the trimolecular complex which triggers disease. However, the usage of peptides as therapeutic entities even when they are strong suppressors of disease in animal models is hindered due to their sensitivity to proteolytic enzymes. Continuous injections and therefore prohibitive amounts of peptides are necessary to elicit the necessary biological response. To address the need for a more stable molecule with the same biological activity for clinical purposes, it is necessary to pursue either the design of cyclic peptides which are more resistant to proteolytic hydrolysis[11] or the design of non-peptide mimetics of the parent peptide with obvious advantages in terms of synthesis cost and oral activity (drug delivery by mouth rather than by injection) compared to the active peptides.[12] A third approach could be a combination of the two methods by incorporating an organic spacer within a cyclized peptide ring. In this study, we designed, synthesised, and evaluated for their activity in EAE assays, cyclic analogues containing aminocaproic acid (Acp) as the spacer group. Cyclization is known to restrict the number of possible conformations of the parent peptide and is an important tool for studying conformation. The intermediate level compounds synthesized are called cyclic semi-mimetic peptides. Some of the compounds synthesized in this study were based on residues of copolymer I (a mixture of random polymers of Ala, Gln, Lys and Tyr) which has proved effective in the treatment of MS.

Results

Scheme 1 shows the synthesis of linear peptide analogue 1 by Fmoc/tBu methodology using the 2-chlorotrityl chloride resin. Scheme 2 shows the synthesis of cyclic analogues **1**–**4**. Figure 1 shows the ability of compounds to induce EAE in Lewis rats. Figure 2 shows the modulatory effect of [Arg^{91},Ala^{96}]MBP$_{87–99}$ and four cyclic



Scheme 1. Synthetic procedure for the linear analogue [Arg^{91},Ala^{96}]MBP$_{87–99}$.



Scheme 2. Synthetic procedure for cyclic analogues **1**, **2**, **3** and **4**.

analogues on EAE upon co-injection with $MBP_{72–85}$. The linear [Arg^{91},Ala^{96}]$MBP_{87–99}$ prevents the development of disease while the four cyclic analogues have no inhibitory effect but delay the onset of disease.

Chemistry

The synthesis of the linear peptide antagonist [Arg^{91}, Ala^{96}]$MBP_{87–99}$, as well as of the cyclic analogues **1**–**4** was carried out by the Fmoc/tBu methodology, utilizing the 2-chlorotrityl chloride resin that was used previously for the synthesis of novel cyclic amide-linked analogues of angiotensins II and III[13] as well as of the thrombin receptor SFLLR motif.[14,15] 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.[14–17] The purification was achieved using HPLC reversed-phase chromatography and the peptide purity was assessed by analytical HPLC and by mass spectrometry (ESIMS).[18]

Inhibitory activity of linear [Arg^{91}, Ala^{96}] $MBP_{87–99}$ in the EAE assay

Immunization of Lewis rats with the human $MBP_{87–99}$ epitope resulted in mild EAE with a peak clinical score of 1 (data not shown). The guinea-pig $MBP_{72–85}$ peptide (30 μg) induced an acute monophasic disease with a peak clinical score of 4 at day 13 after the initial injection, followed by complete recovery by day 18 in all animals injected. The strong and consistent EAE response induced by the guinea-pig peptide was therefore chosen for subsequent analysis of antagonist peptides. Coinjection of [Arg^{91},Ala^{96}]$MBP_{87–99}$ (500 μg) with the potent agonist peptide $MBP_{72–85}$ (30 μg) completely prevented the development of EAE (Fig. 2) demonstrating that this linear antagonist is a potent inhibitor of disease induced by linear analogue $MBP_{72–85}$.

Modulatory activity of cyclic analogues on EAE induced by $MBP_{72–85}$

Co-injection of any cyclic analogue (500 μg) with $MBP_{72–85}$ (30 μg) did not inhibit EAE activity. However, the conjection of all cyclic analogues except cyclo-FARQAcp with $MBP_{72–85}$ resulted in delay of onset of EAE (from day 13 to day 15) but not alteration of its severity (Figs 1 and 2).

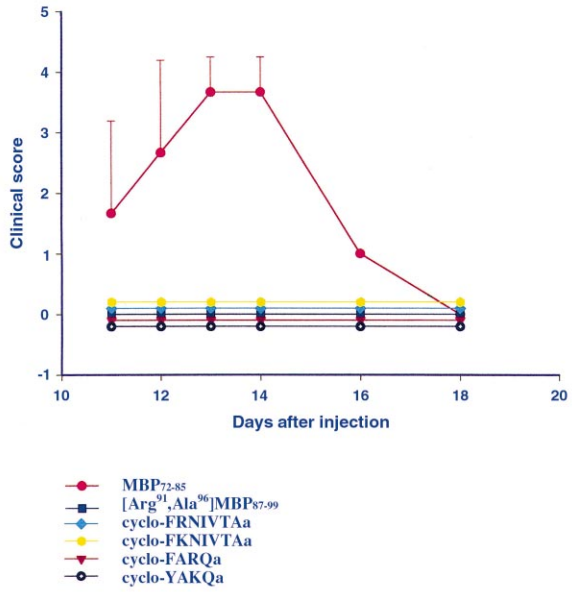


Figure 1. Induction of EAE by $MBP_{72–85}$, $[Arg^{91},Ala^{96}]MBP_{87–99}$ and cyclic analogues.

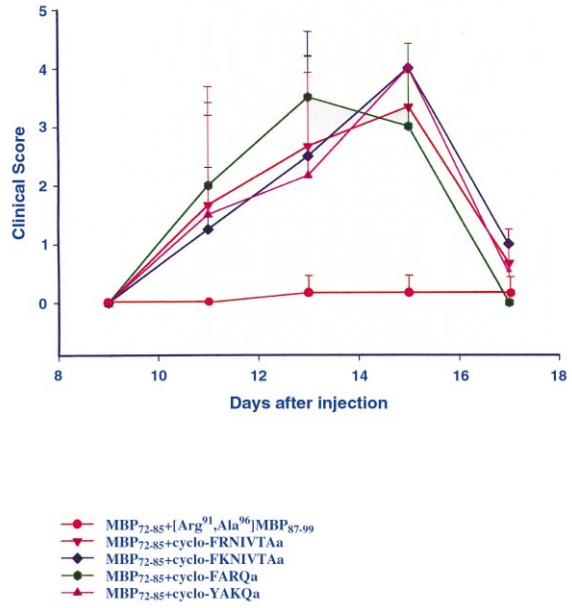


Figure 2. Prevention of $MBP_{72–85}$ induced EAE by $[Arg^{91},Ala^{96}]MBP_{87–99}$ and delay of EAE onset by cyclic analogues.

Molecular modelling of $MBP_{72–85}$, $[Arg^{91},Ala^{96}]MBP_{87–99}$ and cycloPhe-Arg-Asn-Ile-Val-Thr-Ala-Acp

Structural and conformational comparisons of peptides were carried out by energy minimization calculations using HYPERCHEM 5.1 PROFESSIONAL (Hypercube Inc., FL) software run on an Intel workstation. Geometry optimization was initially conducted using molecular dynamics simulations with molecular mechanics (AMBER) minimizations (Polak-Ribiere). Preferred structures were then refined using semiempirical AM1 energy calculations. Overlay of structures and electrostatic potential maps (Fig. 3) using CHEMPLUS software was used to calculate RMS values of fit for pairs of peptides. The level of superimposition for each pair of molecules tested was RMS = 7.61 for $MBP_{72–85}$ and $[Arg^{91},Ala^{96}]MBP_{87–99}$, RMS = 7.35 for $MBP_{72–85}$ and MBP_{87-99}, compared to RMS = 1.31 for $MBP_{87–99}$ and $[Arg^{91},Ala^{96}]MBP_{87–99}$, illustrating that molecules derived from different epitopes of MBP share no similar properties/motifs and would be unlikely to interact with a common receptor.

Discussion

The possibility that $MBP_{87–99}$ has an important role in the pathogenesis of EAE and MS stems from a number

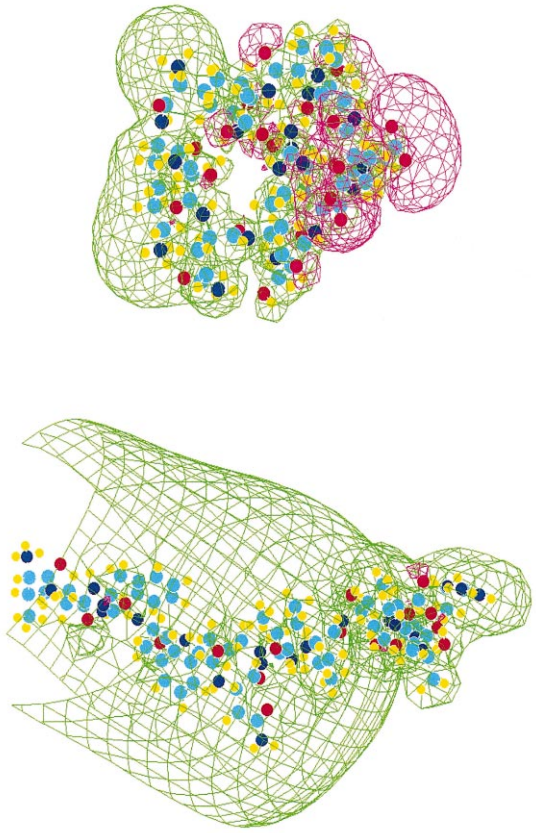


Figure 3. Electrostatic potential 3-D maps for $MBP_{72–85}$ (top) and $[Arg^{91},Ala^{96}]MBP_{87–99}$ (bottom) calculated as described in Results section. Atoms are color coded: carbon (blue), hydrogen (yellow), oxygen (red), nitrogen (blue–black). Grid lines represent electrostatic surfaces: positive (green), negative (red).

of findings. MBP peptides (89–101) and (87–99) are encephalitogenic in rodent strains susceptible to acute (Lewis rats) and chronic (SJL mice) EAE.[19] Furthermore, peptide analogues based on immunodominant epitopes of MBP and PLP can prevent and treat EAE.[20] Moreover, MBP is one of the candidate autoantigens in MS and $MBP_{87–99}$ peptide represents the immunodominant region in the context of human MS associated DR alleles.[1,21,22]

Structure–activity studies have shown that the human $MBP_{87–99}$ peptide induces EAE and that replacement of either Lys with Arg at position 9, or Pro with Ala at position 96, results in analogues with inhibitory effects.[8] In this work, we replaced both residues Lys and Pro of human $MBP_{87–99}$ with Arg and Ala and the resulting analogue completely suppressed the symptoms of EAE induced by $MBP_{72–85}$. The ability of the human [Arg^{91}, Ala^{96}]$MBP_{87–99}$ analogue to inhibit the disease induced by the structurally unrelated epitope $MBP_{72–85}$ is quite intriguing. The mechanism of inhibition could perhaps involve competition of two peptides for binding to the same sites of MHC molecules, although previous work with altered peptide ligands (APL) suggests that the interaction is more likely due to competition at T cell receptor (TCR) sites. However, in the present case there is no sequence homology between the two peptides in question and, despite the fact that there is clearly flexibility of the specificity of TCR recognition, the absence of any perceivable structural relationship at any level between the two peptides, as determined by computer assisted molecular modelling and overlay (Fig. 3), makes it unlikely that they interact with the same TCR. Thus, classical receptor pharmacological concepts of antagonism and desensitization, which normally involve binding interactions at the same receptor molecule, do not provide an adequate explanation for the observed effects.

In another study involving agonist-induced EAE which was suppressed by an APL antagonist, T cell subpopulations were detected for both peptides,[6] raising the possibility that APL might inhibit disease through the activation of antigen specific regulatory T cells. Accordingly-antagonist specific T cells may downregulate agonist specific T cells. Moreover, cross-talk between T cell subpopulations may occur not only for structural variants (APL) of the same epitope[6] but also, as observed in the present investigations, for different epitopes within a given protein antigen. Thus, T cells specific for one epitope may down regulate T cells specific for another epitope within the same protein, creating a dominant subpopulation of T cells for a given protein antigen. This would be in keeping with the observation that polyclonal antibodies raised against a protein or peptide antigen often show remarkable specificity characteristics resembling those of monoclonal antibodies, presumably because of the presence of a dominant subpopulation of antibodies directed towards one particular epitope or motif. Factors which determine 'motif dominance' are not understood, although in our experiments this could have resulted from the different concentrations of the presenting antigens.

Since peptide therapy is hindered due to the sensitivity of peptides to proteolytic enzymes, we attempted to transfer these inhibitory modifications to two cyclic analogues containing the central segment Phe-Lys-Asn-Ile-Val-Thr-Pro of human $MBP_{87–99}$. In cyclic analogue c-Phe-Arg-Asn-Ile-Val-Thr-Ala-Acp both Lys/Pro residues important for activity have been replaced by Arg/Ala residues known to produce inhibitory effects. In cyclic analogue c-Phe-Lys-Asn-Ile-Val-Thr-Ala-Acp, only the Pro residue has been replaced by Ala and the cyclization was carried out by the side chain of Lys and the c-terminous. Aminocaproic acid (Acp) was used as the spacer/linker group incorporated in each linear precursor peptide resulting in a constrained cyclic moiety. Both octapeptide cyclic analogues were ineffective in inducing EAE, or suppressing EAE induced by encephalitogenic $MBP_{72–85}$. In previous work we were able to produce a potent cyclic analogue of $MBP_{72–85}$[11,12] by connecting the Lys and Glu residues at positions 2 and 9 of the dodecapeptide, leaving intact the two N- and C-termini. The findings of this research together with the previous results could illustrate the importance of molecule length, ring size, and availability of termini domains for proper binding to the MHC groove, or for other aspects of T cell regulation.

We also attempted the synthesis of smaller cyclic analogues: (1) c-Tyr-Ala-Lys-Gln-Acp, which incorporates the four amino acids present in Copolymer I (a mixture of random polymers of Ala, Gln, Lys and Tyr) which is used to treat MS,[23] and (2) c-Phe-Ala-Arg-Gln-Acp, which is a variation of 1. These analogues were also ineffective in inducing or suppressing EAE, perhaps validating the proposition that proper size and conformation is required for immunogenic effects.

The development of alternative molecules that will mimic the immunomodulatory activity of MBP epitope peptides and will maintain an advantage over regular peptides in terms of stability is quite challenging, but is a necessary step before these molecules can be used for therapeutic purposes in multiple sclerosis. Presently, several drug design approaches are pursued in different laboratories using combinatorial techniques which may lead to non-peptide mimetics or semimimetic peptides with immunomodulatory activity.[11,12,24,25]

Experimental

Chemistry

Synthesis of linear peptide [Arg^{91},Ala^{96}]$MBP_{87–99}$. The linear peptide was prepared on a 2-chlorotrityl chloride resin 0.7 mmol Cl^-/g using novel solid-phase peptide synthetic methods.[12,26,27] The first N^{α}-Fmoc (9-fluorenylmethyloxycarboxyl)-protected amino acid (Fmoc-Pro-OH (1 equiv)) was coupled to the resin in 1 h in the presence of diisopropylethylamine (DIPEA) (3.2 equiv) in dichloromethane (DCM). The remaining peptide chain was assembled by sequential couplings of the following Fmoc protected amino acids (2.5 equiv): Fmoc-Thr (tBu)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Ala-OH, Fmoc-

Val-OH, Fmoc-Ile-OH, Fmoc-Asn-OH, Fmoc-Phe-OH, Fmoc-His(Trt)-OH in the presence of *N*,*N*′-diisopropylcarbodiimide (DIC) (3.75 equiv) and 1-hydroxybenzotriazol (HOBt) (3.5 equiv) in *N*,*N*-dimethylformamide (DMF) for 4 h. The completeness of each coupling was verified by the Kaiser test and TLC. The Fmoc protecting groups were removed by treatment with piperidine (20% in DMF, 2×10 min). The protected peptide resin was then cleaved with the splitting solution dichloromethane: acetic acid: 2,2,2-trifluoroethanol (DCM:HOAc:TFE, 7: 1:2 1 h at room temperature). The solvent was removed on a rotary evaporator and the obtained oily product precipitated from cold dry diethylether as a white solid. The deprotection of protected linear peptides was achieved with 65% trifluoroacetic acid (TFA) in DCM in the presence of 1,2 ethanedithiol (EDT) while stirring for 4 h.

Synthesis of cyclic semimimetic peptides: cyclo-Phe-Arg-Asn-Ile-Val-Thr-Ala-Acp (1), cyclo-Phe-Ala-Arg-Gln-Acp (2), cyclo-Tyr-Ala-Lys-Gln-Acp (3), cyclo-Phe-Lys-Asn-Ile-Val-Thr-Ala-Acp (4)

(a) Synthesis of precursor protected linear peptides. This synthesis has been accomplished using Fmoc/tBu solid phase methodology and the acid sensitive 2-chlorotrityl resin using methods previously described.[12,26,27] Fmoc-Acp-OH for each semi-mimetic peptide was attached to the resin by a simple, fast and racemization free reaction using (DIPEA) in (DCM) solution at room temperature. Stepwise synthesis of the protected linear peptides was achieved with *N*,*N*′-diisopropylcarbodiimide/1-hydroxybenzotriazol (DIC/HOBT) as coupling agent. In particular, Boc-Phe-OH and Fmoc-Lys(Mtt)-OH were used for the synthesis of cyclic peptide **4**. The cleavage of peptide from the resin and the Mtt deprotection was achieved with the splitting solution DCM:HOAc:TFE, 7:1:2 which allows the acid sensitive protecting groups tBu (for Thr), tBu (for Tyr), Pbf (for Arg), Boc (for Lys), Boc (for N^{a}Phe) to remain intact. The prepared precursor peptides were precipitated upon addition of ether (Scheme 2).

(b) Cyclization of protected peptides. To a solution which contains each of linear protected peptide in dry DMF was added 2,4,6-collidine and 1-hydroxy-7-azabenzotriazole were added. The solution was added dropwise to a solution of *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU) in dry dimethylformamide (DMF) for 2 h, and the solution was stirred for 3 h.[11,14] 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_2O and was dried in vacuum for 12 h. Removal of the protecting groups (Thr-tBu, Arg-Pbf, Tyr-tBu, Lys-Boc and N^{a}Phe-Boc) was achieved by treatment of the protected peptides with TFA/CH_2Cl_2 (65%, 10 mL/g) in the presence of 1,2 ethanedithiol as scavenger (Scheme 2).

HPLC/TLC/ESIMS. Preparative HPLC for linear and cyclic $MBP_{87–99}$ analogues was performed with a Waters system equipped with a 600E system controller using a Lichrosorb RP-18 reversed-phase preparative column (250×10 mm) with 7 μm packing material.[19,20] Separations were achieved with a stepped linear gradient of acetonitrile (AcN) (0.08%TFA) in water (0.08% TFA) over 60 min at a flow rate of 3 mL/min. The crude peptide material (20 mg) was dissolved in methanol/water (450 μL), and this solution was injected using a Waters U6K injector with a 2.0 mL sample loop. Elution of the peptide was determined simultaneously from the absorbances at 230 and 214 nm (Waters 996 photodiode array detector). Fractions containing the major peptide peak were pooled and acetonitrile was removed using a rotary evaporator. After lyophilization the product was stored at −20 °C. Peptide purity was assessed by analytical HPLC reruns (Nucleosil-120 C18, 250×4.0 mm), thin layer chromatography (TLC) and mass spectrometry (ESIMS). The TLC solvent systems were used as follows: *n*-butanol:acetic acid:water (4:1:1) (BAW), toluene:acetic acid:-methanol (7:1.5:1.5) (TAM), *n*-butanol:pyridine:acetic acid:water (15:10:3:6) (BPAW). The ESIMS spectra were run on a TSQ 7000 Spectrometer (Electrospray mode) by direct infusion. A solution of the sample (1 μg:1 μL) in methanol was introduced in the ESI probe at a flow rate 5 μL/min with a Harvard syringe. The capillary temperature was at 80 °C and the sheath gas at 45 units whilst the spray needle voltage was + 4.5 KV.

Induction or Suppression of EAE by $MBP_{72–85}$ and [Arg^{91}, Ala^{96}]$MBP_{87–99}$. Female Lewis rats (220 g) were immunized with $MBP_{72–85}$ (30 μg), [Arg^{91},Ala^{96}]$MBP_{87–99}$ (500 μg) or each of the four cyclic semi-mimetic peptide (500 μg) (n = 4 per group). Furthermore [Arg^{91},Ala^{96}] $MBP_{87–99}$ (500 μg) and each of the four cyclic semi-mimetic peptides (500 μg) were co-injected with $MBP_{72–85}$ in Lewis rats. Peptides were dissolved in PBS and emulsified in an equal volume of Freunds complete adjuvant (CFA, Difco) containing 4 mg/mL heat-killed *Mycobacterium tuberculosis* H37Ra (Difco). Immunization was performed by subcutaneous injection, in the two hind footpads, with 200 uL of an emulsion consisting of the peptide dissolved in 0.1 mL of PBS, and 0.1 mL Freunds complete adjuvant (FCA) containing 4 mg/ml heat-killed *Mycobacterium tuberculosis* (H37Ra) (Difco). Rats were weighed and examined for clinical signs daily. Clinical EAE was graded on a scale of 0–4 by established criteria as follows: 0, no discernable disease; 0.5, weight loss; 1, flaccid tail; 2, hind limb weakness; 3, paraplegia; 4, paraplegia with forelimb weakness, moribund.

Acknowledgements

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