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Design and synthesis of small semi-mimetic peptides with immunomodulatory activity based on Myelin Basic Protein (MBP)

T. Tselios[1], L. Probert[2], G. Kollias[2], E. Matsoukas[1], P. Roumelioti[1], K. Alexopoulos[1], G. J. Moore[3], and J. Matsoukas[1]

[1] Department of Chemistry, University of Patras, Patras, Hellas
[2] Department of Molecular Genetics, Hellenic Pasteur Institute, Athens, Hellas
[3] Pharmacology and Therapeutics, Health Science Center, University of Calgary, Calgary, Alberta, Canada

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Summary. Experimental allergic encephalomyelitis (EAE) is induced in susceptible animals by immunodominant determinants of myelin basic protein (MBP). Analogs of these disease-associated peptides have been identified with disease progression upon coimmunization. Usage of peptides, with disease-specific immunomodulatory capacity in vivo is limited, however, due to their sensitivity to proteolytic enzymes. Alternative approaches include the development of mimetic molecules which maintain the biological function of an original peptide, yet are stable and able to elicit their response in pharmacological quantities. A novel technique was employed to design a series of semi-mimetic peptides, based on the guinea pig MBP_{72-85} peptide used to induce EAE in Lewis rats. We used isonipecotic (iNip) and aminocaproic (Acp) acids as templates. Acp-MBP_{72-85} peptide derived analogues were effective in inducing EAE compared to iNip-peptide analogues which were ineffective at 350μg. These findings suggest that the design and synthesis of semi-mimetic peptide molecules with immunomodulatory potential is possible and that eventually these molecules may form the basis for the development of novel and more effective disease-specific therapeutic agents

Keywords: Amino acids – Experimental allergic encephalomyelitis (EAE) – Myelin basic protein (MBP) – Semi-mimetic peptides

Introduction

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) characterized by focal T cell and macrophage infiltrates, demyelination and loss of neurologic fuction (Martin et al., 1992). 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 therapeutical management of MS involve the design and use of peptide analogues of disease-associated myelin epitopes to induce peripheral T cell tolerance. Experimental autoimmune encephalomyelitis (EAE) one of the best studied experimental animal models of MS (Zanvil et al., 1990), represents an invaluable in vivo system for the evaluation of such therapeutic approaches. EAE is a CD4+ T cell-mediated disease that can be induced by immunisation with MBP and PLP proteins and 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 (Marca et al., 1992; Chou et al., 1989; Wucherpfennig et al., 1994).

The assumption has been that disease can be modulated with peptides that interfere with the formation of the trimolecular complex MHC-Peptide-T cell receptor. Peptide therapy, however, is hindered due to the sensitivity of peptides to proteolytic enzymes. Continuous injections and therefore prohibitive amounts of peptides are necessary to elicit the necessary biological response. To address the need for more stable molecules with the same biological activity, a new technology has been developed that generates organically synthesized molecules called mimetics (Moore et al., 1995; Matsoukas et al., 1994). Several approaches have been used for the development of mimetic molecules including random pharmacological screenings of synthetic molecules, combinatorial small molecule libraries and natural products. The novel techology applied here involves mounting the essential functional amino acids derived from a bioactive peptide onto the arms of a "molecular hinge", which greatly facilitates the opportunity for the bioactive residues to cluster together (closed hinge) or otherwise (open hinge) in an appropriate manner. In this work we used Isonipecotic acid and Aminocaproic acid as templates which allow either constrained orientation of the pharmacophoric groups or extension of the peptide chain to the desired length for maximum binding.

Methods

Synthesis of peptide analogues of MBP_{72-85}: H-Gln-Lys-Ser-Gln-Arg-Ser-Gln-X-Glu-Asn-Pro-Val-OH: X 5 Ala, X 5 Asp

Fmoc-Val-2-chlorotrityl resin was used for the synthesis of the linear peptide precursors, following the protocol previously described (Matsoukas et al., 1994; Barlos et al., 1989). The amino acids used in Fmoc synthesis were: Fmoc-Val-OH, Fmoc-Pro-OH, Fmoc-Asn-OH, Fmoc-Ala-OH, Fmoc-Arg(Pmc)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Asp(tBu)-OH, Fmoc-Glu(tBu)-OH, Fmoc-Gln-OH, Fmoc-Ser(tBu)-OH. The finished peptide-resin (after Fmoc-deprotection of the last amino acid) was dried in vacuo and then was treated with the splitting mixture dichloromethane/acetic acid/2, 2, 2 trifluoroethanol (7:1:2) for 1 h at room temperature to remove the peptide from the resin. The mixture was filtered off and the resin washed again with the splitting mixture (×2) and DCM (×3). The solvent was removed on a rotary evaporator and the obtained oily product precipitated from cold dry diethylether as a white solid. Deprotection, using 65% TFA +3% ethanedithiol in DCM for 4 h (Sheme 1) afforded the final free peptides which were purified by preparative HPLC.

Synthesis of semi-mimetic peptides: H-Ser-Arg-iNip-Acp-Glu-NH_2, H-Ser-Arg-iNip-Acp-Acp-Glu-NH_2, H-Ser-Arg-Acp-Acp-Glu-NH_2, H-Ser-Arg-Acp-Acp-Acp-Glu-NH_2

Fmoc-Glu(tBu)-Linker-2-chlorotrityl resin was used for the synthesis of the semi-mimetic peptide precursors, following the protocol previously described (Matsoukas et al., 1994; Barlos et al., 1989). The amino acids used in Fmoc synthesis were: Fmoc-Arg(Pmc)-OH, Fmoc-iNip-OH, Fmoc-Acp-OH, Fmoc-Glu(tBu)-OH, Fmoc-Ser(tBu)-OH. The finished peptide-linker (after cleavage from the resin and Fmoc deprotection of the last amino acid) was treated with 65% TFA + 3% ethanedithiol in DCM for 4 h to afford free semi peptide. (Sheme 2).

Preparative HPLC 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. Separations were achieved with a stepped linear gradient of acetonitrile (0.1% TFA) in water (0.1% TFA) over 60 min at rate of 3 ml/min. The crude peptide material (20 mg) was dissolved in methanol/water (450 ml) 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 254 and 230 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.

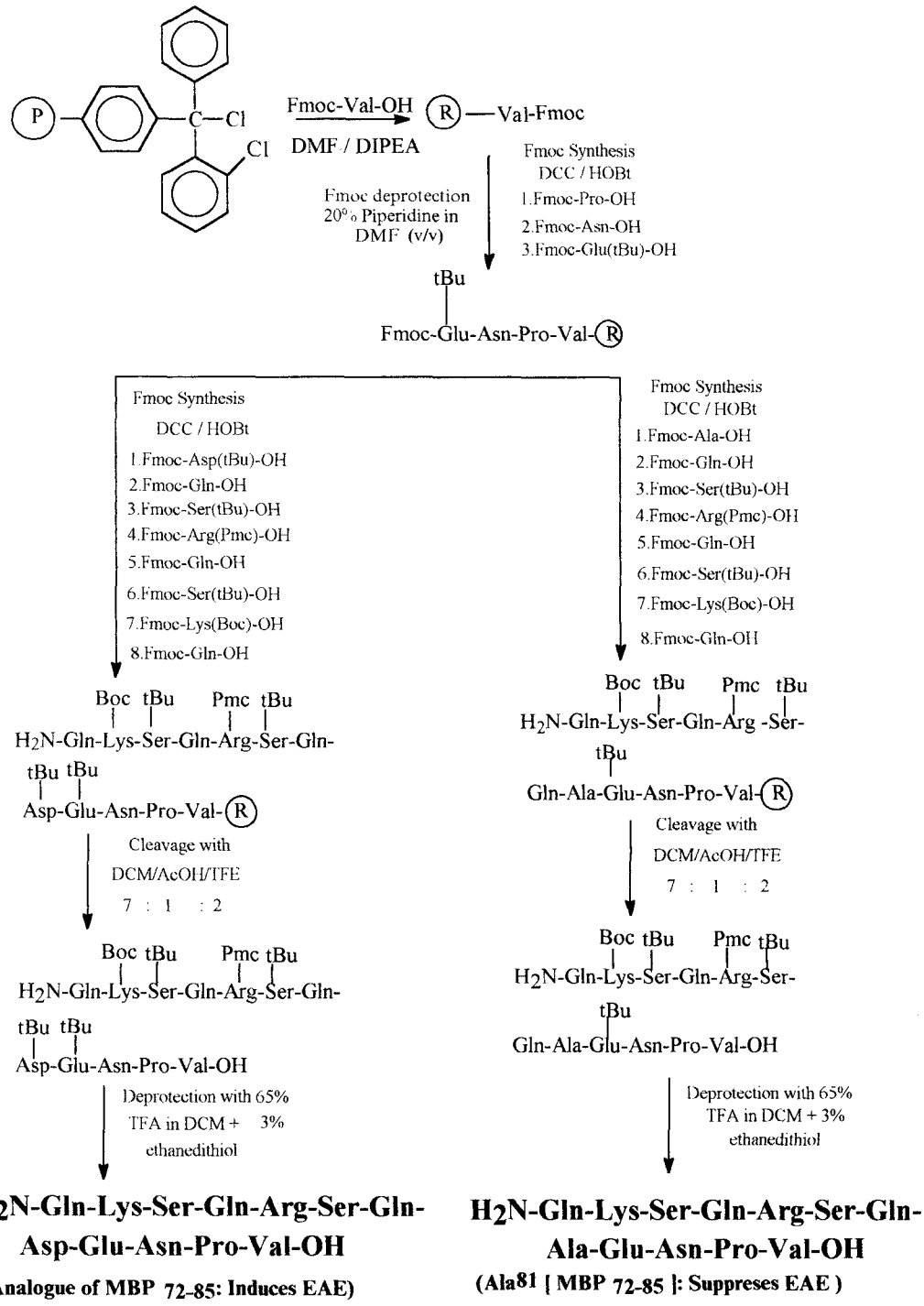
Peptide purity was assesed by analytical HPLC reruns (Noucleosil C 18, 250 × 4.0 mm), thin layer chromatography (TLC), and mass spectrometry (FABMS, ESIMS). Two TLC solvent systems were used as follows: n-butanol/acetic acid/water (4:1:1) (BAW), toluol/methanol/water (70:15:15) (TMW) and n-butanol/pyridine/acetic acid/water (15:10:3:12) (BPAW).

Induction of EAE

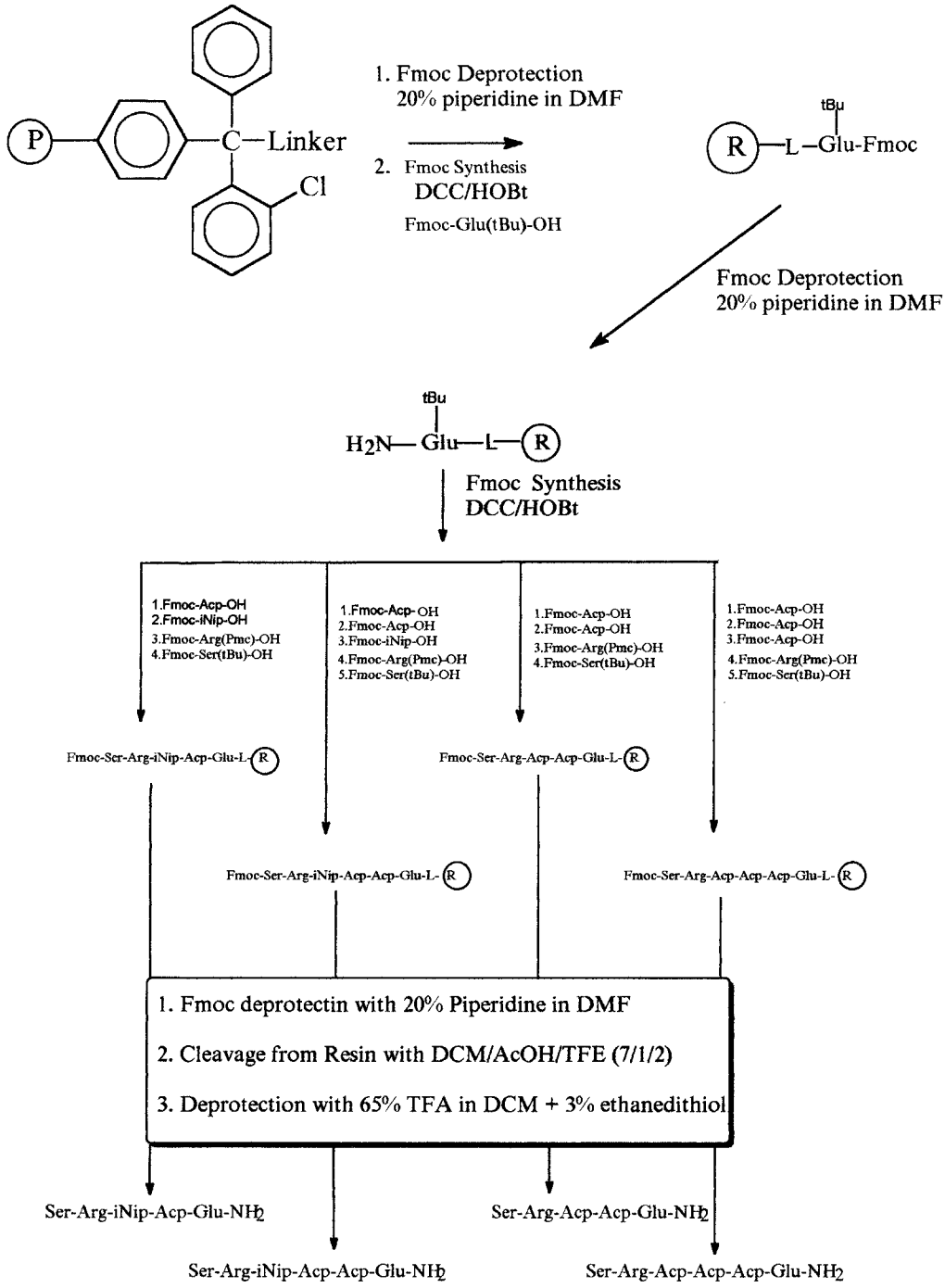
Female Lewis rats (200 g) were immunized subcutaneously into the hind footpads with MBP_{72-85} (25 μg) (n = 10), MBP_{72-85} (25 μg) plus Ala^{81} MBP_{72-85} (500 μg) (n = 2) or semi-mimetic peptide (at 100 μg, 350 μg, 500 μg and 1 mg per dose per animal) (n = 4 per group). Peptides were dissolved in non-pyrogenic water and emulsified in an equal volume of Freund's complete adjuvant (Difco, USA) containing 4 mg/ml heat-killed Mycobacterium tuberculosis H37Ra (Difco). Each injection contained peptide at the doses shown above, with 400 μg Mycobacterium tuberculosis in a volume of 200 μl. The injection was repeated 7 days following the initial injection. Clinical EAE was graded on a scale of 1–4 by established criteria as follows; 0, no clinical disease; 0.5, weight loss; 1, tail weakness; 2, mono- or paraparesis (incomplete paralysis of one or two hindlimbs); 3, mono- or paraplegia (complete paralysis of one or two hindlimbs); 4, paraplegia with forelimb weakness or paralysis, moribund.

Results

To evaluate whether this technology can provide molecules with the immunomodulatory ability displayed by the actual peptides derived from MBP, the analogue of 72-85 amino acid sequence of guinea pig MBP was used as a reference peptide. It has been reported that this peptide induces EAE in Lewis rats and that single alanine-substituted peptide analogs at positions K^{74},S^{75},R^{78},Q^{80},D^{81},E^{82} and P^{84} resulted in significant reduction of the proliferative responses of a T cell line that is specific for the MBP_{72-85} peptide. Furthermore the Ala^{81} analogue, when coinjected with analogue of MBP_{72-85} peptide,



Scheme 1. Synthesis of MBP_{72-85} peptides



Scheme 2. Synthesis of MBP_{72-85} derived semi-mimetic peptides

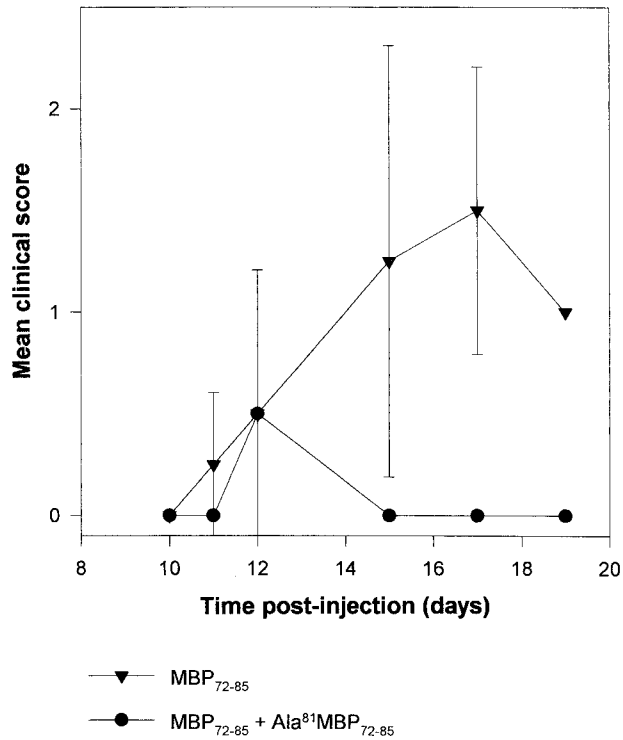


Fig. 1. Prevention of MBP_{72-85} induced EAE by analogue $Ala^{81}MBP_{72-85}$. EAE was induced by footpad injection of 25μg MBP_{72-85} (▼) and prevented by the co-injection of 25μg MBP_{72-85} plus 500μg of the peptide analogue $Ala^{81}MBP_{72-85}$ (●)

suppressed EAE (Fig. 1). Since the peptides that bind on an MHC molecule have been determined to involve a minimum of nine amino acid residues which satisfy a particular motif, the design of mimetics that would require a reduced number of amino acids and still maintain their funtional role in vivo is quite challenging. The participation of moieties however, like iNip that represents a length of aproximately 1.5 peptidic bonds and provides restriction or flexibility to the whole peptide, as it assumes all intermediate structures between "chair" and "boat" conformation, introduces parameters that have not been tested previously for their effect in the immune response. Amino acids Ser, Arg, iNip, Acp and Glu were used in different combinations to synthesize semi-mimetic peptides that could possibly reproduce the activity of the analogue of MBP_{72-85} peptide. In contrast to other attempts that employ random screening of synthetic organic molecules or combinational libraries, this is an attempt to explore how rationally synthesized compounds, which are based on peptide sequence that induces a particular immune response (EAE), may mimic its biological activity.

Semi-mimetic peptides synthesized and their basic characteristics

The synthesized semi-mimetic peptides are presented in Fig. 2. Characteristically, with Ser, Arg and Glu amino acids, they also include the iNip and Acp

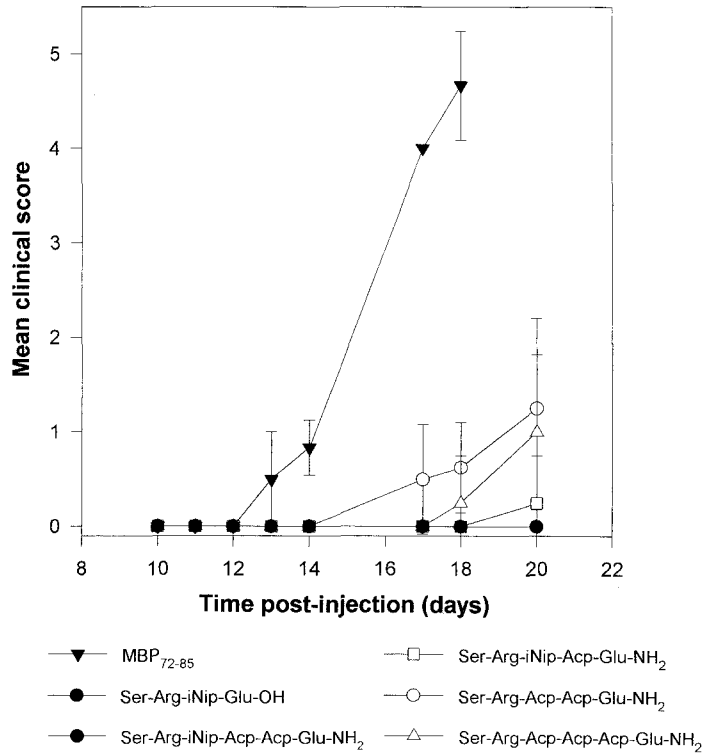


Fig. 2. Induction of EAE by MBP_{72-85} semi-mimetic peptides. EAE was induced by footpad injection of 25μg MBP_{72-85} as positive control, and by 350μg each of the semi-mimetic peptides Ser-Arg-Acp-Acp-Glu-NH_2 (●) and Ser-Arg-Acp-Acp-Acp-Glu-NH_2 (△). Semi-mimetic peptide, Ser-Arg-iNip-Glu-OH, induced low-level EAE only when injected at much higher concentrations (1mg per animal) (not shown here). Clinical disease induced by the semi-mimetic peptides was significant when compared to normal controls by the Student's t test, $p < 0.05$

Table 1. Average day of onset of EAE in rats immunized with analogue MBP_{72-85} or semi-mimetic peptides (350μg)

Immunization	Average day of onset	P value
MBP_{72-85}	12.5	$p < 0.00001$
MBP_{72-85} + $Ala^{81}MBP_{72-85}$	no EAE	
Ser-Arg-iNip-Glu-OH	no EAE	$p > 0.350$
Ser-Arg-iNip-Acp-Glu-NH_2	no EAE	
Ser-Arg-iNip-Acp-Acp-Glu-NH_2	no EAE	
Ser-Arg-Acp-Acp-Glu-NH_2	17	$p = 0.040$
Ser-Arg-Acp-Acp-Acp-Glu-NH_2	17	$p = 0.049$

residues that render the peptide constrained or flexible. Each of the semi-mimetic peptides (Table 1, Fig. 2) and the reference peptide analogue of MBP_{72-85} were injected individually in Lewis rats and tested for disease induction. MBP_{72-85} consistently induced severe EAE with clinical onset between

days 12–13 post-injection when injected at 25μg per animal (Fig. 1). When the altered peptide ligand Ala^{81}MBP$_{72-85}$ was co-injected at 500μg per animal together with the encephalitogenic MBP$_{72-85}$ peptide, the onset of clinical disease was completely prevented in all animals tested (Fig. 1).

Effect of semi-mimetic peptide on disease (EAE) compared with MBP$_{72-85}$ analogue

The aminocaproic acid-extended peptides Ser-Arg-Acp-Acp-Glu-NH_2 and Ser-Arg-Acp-Acp-Acp-Glu-NH_2 were effective in inducing clinical EAE at a dose of 350μg per animal with delayed clinical onset when compared to MBP$_{72-85}$ (Table 1, Fig. 2). The Isonipecotic-containing peptides appeared to be less effective in inducing clinical EAE. At the same dose of 350μg per animal only Ser-Arg-iNip-Acp-Glu-NH_2 induced a low grade disease by day 18 post-injection. At higher doses of 500μg and 1 mg per animal Ser-Arg-iNip-Glu-OH also induced disease with a maximal clinical score of 2 and onset between days 19–21 post-injection.

Discussion

Development of alternative molecules that will mimic the immunomodulatory activity of peptides and will maintain an advantage over regular peptides in terms of stability and cost is a necessary step, before peptides can be used for therapeutic purposes. In this report, preliminary evidence is presented suggesting that, a novel technique that has been used successfully for the development of mimetic molecules of angiotensin, vasopressin and gonadropine-releasing hormone, may eventually be applicable in generating mimetic molecules of peptides with immunomodulatory capability (Moore et al., 1995). The guinea pig analogue of MBP$_{72-85}$ peptide that induces transient EAE in Lewis rats, was used as a reference peptide to synthesize semi-mimetic molecules. The novel method employed for this purpose involves the participation of iNip and Acp two moieties that provide rotational constriction or flexibility to the peptide. Binding of small peptide molecules with the conformation dictated by iNip and Acp has not been studied previously for possible immunomodulatory activity.

In this study we have found that iNip-MBP$_{72-85}$ derived analogues (Ser-Arg-iNip-Glu-OH, Ser-Arg-iNip-Acp-Glu-NH_2, Ser-Arg-iNip-Acp-Acp-Glu-NH_2) were not active at 350μg in inducing EAE effect. However, Acp-MBP$_{72-85}$ derived analogues (Ser-Arg-Acp-Acp-Glu-NH_2 , Ser-Arg-Acp-Acp-Acp-Glu-NH_2) induced significant EAE at 350μg, ($p = 0.040$ and $p = 0.049$) indicating steric preferences for maximum activity of the semi-mimetic analogues. The isonipecotic ring induces a constrained conformation in which the pharmacophoric groups of Ser, Arg, Glu are obliged to cluster together or to be on opposite sites in the boat or chair conformation of the piperidine ring. On the other hand the Acp five carbon chain linear template induces a relaxed conformation in which the pharmacophoric groups are far apart being possibly closer to the receptor pockets.

This information may be important for designing more effective semi-mimetic analogues or mimetics based on MBP_{72-85} .

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References

Marca B, Wauben HM, Boog CJP, Zee R, Joosten I, Scihlief A, Eden W (1992) Disease inhibition by major histocompatibility complex binding peptide analogues of disease-associated epitopes: more than blocking alone. J Exp Med 176: 667–677

Chou YK, Vandenbark AA, Jones R, Hashim G, Offner H (1989) Selection of encephalitogenic rat T lymphocyte clones recognizing an immunodominant epitope on myelin basic protein. J Neuros Res 22: 181

Zamvil SS, Steinman L (1990) The T lymphocyte in experimental allergic encephalomyelitis. Ann Rev Immunol 8: 579–621

Wucherpfennig KW, Sette A, Southwood S, Oseroff C, Matsui M, Strominger JL, Hafler DA (1994) Structural requirements for binding of an immunodominant myelin basic protein peptide to DR_2 isotypes and for its recognition by human T cell clones. J Exp Med 179: 279–290

Moore GJ, Smith JR, Baylis B, Matsoukas JM (1995) Design and pharmacology of peptide mimetics. Ad Pharmacol 33: 91–141

Matsoukas JM, Hondrelis J, Keramida M, Mavromoustakos T, Makrijannis A, Hamdagni R, Wu Q, Moore GJ (1994) Role of NH_2-terminal domain of angiotensin II and [Sar^1] angiotensin II on conformation and activity: NMR evidence for aromatic ring clustering and peptide backbone folding compared to [$Des^{1,2,3}$]-angiotensin II. J Biol Chem 269: 5303–5312

Matsoukas JM, Hondrelis J, Agelis G , Barlos K, Gatos D, Ganter RC, Moore D, Moore GJ (1994) Novel synthesis of cyclic amide-linked analogues of angiotensin II and III. J Med Chem 37: 2958–2669

Martin R, McFarland HF, McFarlin DE (1992) Immunological aspects of demyelinating diseases. Ann Rev Immunol 10: 153–187

Barlos K, Gatos D, Hondrelis J, Matsoukas J, Moore GJ, Schafer W, Sotiriou P (1989) Preparation of new acid-labile resins of sec-alcohol type and their applications in peptide synthesis. Liebigs Ann Chem 951–955

Authors' address: Dr. J. Matsoukas, Department of Chemistry, University of Patras, GR-26500 Patras, Greece.

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