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A Synthetic Glycopeptide of Human Myelin Oligodendrocyte Glycoprotein To Detect Antibody Responses in Multiple Sclerosis and Other Neurological Diseases

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Abstract: Glycopeptides of hMOG(30-50) containing a glucosyl moiety on the side-chains of Asn, Ser or Hyp at position 31 were synthesised. Antibody titres to hMOG(30-50) and to its glucoderivatives were measured by ELISA in sera of patients affected by different neurological diseases. Anti-hMOG(30-50) antibodies were detected only using the glycopeptide [Asn^{31}(*N*-Glc)]hMOG(30-50).

Keywords: MOG; Glycopeptides; Multiple sclerosis; Antibodies

MS[1] is a human demyelinating disease of the CNS whose pathogenesis has not been yet elucidated. An autoimmune mechanism against the prominent CNS myelin Ags is thought to contribute to the immunopathogenesis of this disease, even if the target Ags responsible for this inflammatory and demyelinating response remain elusive.

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Although specific responses to the major myelin Ags such as MBP and PLP are likely to be important in the course of MS, they may not represent the primary auto-Ags triggering the autoimmune response in this disease. It has been recently reported that autoimmune recognition of quantitatively minor myelin components, such as MOG, could play a relevant role in the initiation and progression of MS.

MOG has been detected only in the CNS of mammalians and it accounts for about 0.01-0.05% of total myelin proteins. It is a unique member of the Ig superfamily because it possesses two potential transmembrane domains and one *N*-linked glycosylation site on Asn^{31}, but the nature of the linked glycosyl moiety is still unknown.

MOG exposure on the outermost surface of myelin makes it an ideal target antigen for Ab-recognition. Immunisation with purified or recombinant MOG and corresponding synthetic peptides can induce strong immune responses associated with extensive CNS inflammation and demyelination in rodents.[2] In the common marmoset *Callithrix jacchus* MOG induces a relapsing-remitting form of EAE characterised by extensive plaque-like demyelination similar to the human disease MS.[3] It was previously reported a predominant T cell response to MOG in the peripheral blood of MS patients.[4] In contrast, only few and controversial data about anti-MOG Abs are reported in MS patients and no data are available using MOG peptides as antigens.[5]

Post-translational modifications of proteins, such as glycosylation, may dramatically affect their antigenic properties. Several glycoproteins as, for example, fibronectin expose to Abs discontinuous epitopes that may involve glycosylation sites. There are evidences of Abs recognising neither peptide nor carbohydrate *per se*, but rather they are directed to a specific peptide conformation induced by glycosylation.[6] The difficulty to find MOG Abs in humans could be related to the fact that the human antigen is a glycosylated epitope with a well-defined conformation.

The peptide MOG(35-55) is the immunodominant portion of MOG for both T and B cell responses in different animal strains.[7] Anti-MOG(35-55) Abs in Lewis rats strongly reacted against the sequence 37-46.[8] As position 31 is the unique glycosylation site of native MOG,[9] we decided to synthesise glycopeptides of hMOG(30-50). This peptide, containing the immunodominant epitope and the native glycosylation site, was modified by introducing an *N*- or *O*-linked β-D-glucopyranosyl residue. In first instance, we chose to introduce the glucosyl moiety by different covalent bonds, changing the amino acid in position 31 from an Asn to a Ser and a Hyp, in order to verify whether the Ab-response was influenced only by the increased hydrophilicity of the glycopeptide or by different conformations. In particular, Hyp could provoke a significant change in the final conformation of the glycopeptide, because of its rigid and planar conformation.

Results and Discussion

In order to detect by ELISA[10] the presence of anti-MOG peptide-Abs in sera of patients affected by MS and other neurological diseases,[11] we synthesised the wild-type peptide hMOG(30-50) (MOG1) and three corresponding *N*- and *O*-linked glycopeptides, *i.e.* [Asn^{31}(*N*-Glc)]hMOG(30-50) (MOG2), [Ser^{31}(*O*-Glc)]hMOG(30-50) (MOG3) and [Hyp^{31}(*O*-Glc)]hMOG(30-50) (MOG4), as putative

antigens (Table 1). The three glycopeptides were chosen in term of the different covalent bond between the glucosyl moiety and the side chain of the amino acid introduced in the sequence at position 31. In fact, whereas MOG2 contains an *N*-glucosyl moiety on Asn (amide bond), as in the native protein, MOG3 and MOG4 contain an *O*-glucosyl moiety on a Ser or a Hyp, respectively (ether bond).

Table 1
Amino acid sequences of the hMOG(30-50) peptide derivatives

Peptide	Sequence
MOG1	H-Lys-Asn-Ala-Thr-Gly-Met-Glu-Val-Gly-Trp-Tyr-Arg-Pro-Pro-Phe-Ser-Arg-Val-Val-His-Leu-OH
MOG2	H-Lys-Asn(*N*-Glc)-Ala-Thr-Gly-Met-Glu-Val-Gly-Trp-Tyr-Arg-Pro-Pro-Phe-Ser-Arg-Val-Val-His-Leu-OH
MOG3	H-Lys-Ser(*O*-Glc)-Ala-Thr-Gly-Met-Glu-Val-Gly-Trp-Tyr-Arg-Pro-Pro-Phe-Ser-Arg-Val-Val-His-Leu-OH
MOG4	H-Lys-Hyp(*O*-Glc)-Ala-Thr-Gly-Met-Glu-Val-Gly-Trp-Tyr-Arg-Pro-Pro-Phe-Ser-Arg-Val-Val-His-Leu-OH

Table 2
Chemical data of the hMOG(30-50) peptide derivatives[12,13]

Peptide		Gradients at 3 mL min^{-1} for semi-prep. HPLC	Yield(%)	ESI-MS $[M+H]^+$ Found (Calc.)	R_t (min)[a]
MOG1	hMOG(30-50)	25-40% B in 30 min	12.5	2445 (2444.2)	9.2[b]
MOG2	Asn^{31}(*N*-Glc)hMOG(30-50)	20-45% B in 75 min	10.7	2606 (2605.4)	8.8[b]
MOG3	Ser^{31}(*O*-Glc)hMOG(30-50)	10-60% B in 60 min	18.0	2579 (2578.4)	10.5[c]
MOG4	Hyp^{31}(*O*-Glc)hMOG(30-50)	10-60% B in 60 min	20.0	2606 (2605.4)	8.3[c]

[a]Analytical HPLC gradients at 1 mL min^{-1}: [b]30-40% B in 7 min; [c]10-60% B in 15 min.

Chemistry

Glycosylation of peptides can be achieved directly on the resin,[14] but the building-block approach is the most versatile and general method for the preparation of a large variety of glycopeptides with well-defined and predetermined structures.[15] The syntheses of the new glycopeptides were performed by the solid phase methodology, following the Fmoc/*t*Bu strategy by introducing the three different glucosylated building blocks at position 31. To this aim we synthesised N^{α}-Fmoc-N^{4}-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-Asn-OPfp,[16] N^{α}-Fmoc-O^{3}-(2,3,4,6-tetra-*O*-benzoyl-β-D-glucopyranosyl)-Ser-OPfp[17] and N^{α}-Fmoc-O^{4}-(2,3,4,6-tetra-*O*-benzoyl-β-D-glucopyranosyl)-Hyp-OPfp.[18] All the glucosylated building blocks were purified by FCC (AcOEt/hexane 1 : 1.5) in order to obtain pure β anomers to be used for the synthesis of the glycopeptides (Table 2).

Biology

The Ab titres against MOG1 and its glycoderivatives were compared in the sera of ten patients. No Ab response was detected using the non-glucosylated peptide MOG1. In contrast, Ab reactivity to the glycosylated peptide MOG2 was found in sera of patients affected by MS as well as other neurological diseases (Fig. 1),[10] but not in sera collected from normal blood donors (data not shown). The observation that high Ab titres could be detected by using MOG2, but not MOG3 and MOG4 as antigens (Fig. 1 and Fig. 2), indicated that the Ab response to the glycopeptide MOG2 is not an unspecific phenomenon due to the presence of the glucosyl moiety. These data suggest that the Abs

detected by MOG2 recognise a glycopeptide epitope and that the different covalent bond between the glucosyl moiety and the side chain of the amino acid dramatically affects the Ab recognition.

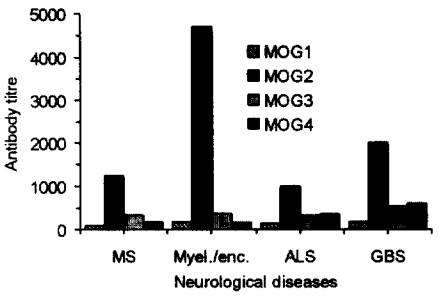

Fig. 1: Ab-titre to hMOG(30-50) and its glycopeptides in sera of: 5 MS, 2 Myel./Enc., 1 ALS and 2 GBS patients. Data are expressed as mean values.

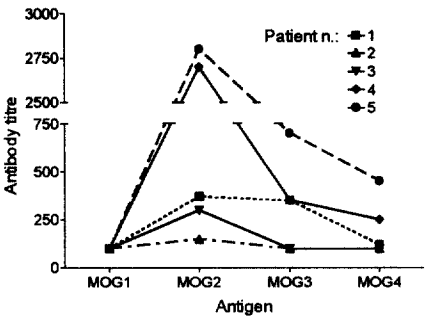

Fig. 2: Ab-titre to hMOG(30-50) and its glycopeptides in sera of 5 MS patients. Data are expressed as mean values.

These results demonstrate, for the first time, the possibility to identify anti-MOG peptide Abs in humans and suggest that the use of the synthetic glycopeptide [Asn^{31}(*N*-Glc)]hMOG(30-50) as an antigen in ELISA may represent a simple experimental system to investigate auto-reactive Ab responses in human diseases of the nervous system. Other MOG peptides or other carbohydrate derivatives may reveal additional epitopes recognised by MS auto-Abs.

In any case [Asn^{31}(*N*-Glc)]hMOG(30-50) is the first synthetic antigen able to detect anti-MOG peptide Abs in 40% of an unselected MS group of patients, as well as in some other neurological diseases. The follow-up of the Ab titre to hMOG(30-50) during the evolution of MS would be helpful to clarify the autoantibody pathogenic role.

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References and notes

1. Abbreviations used: Ab, antibody; AcOEt, ethyl acetate; Ag, antigen; ALS, amyotrophic lateral sclerosis; CNS, central nervous system; CSF, cerebrospinal-fluid; DCM, dichloromethane; DIPEA *N,N*-diisopropylethylamine; DMF, *N,N*-dimethylformamide; EAE, experimental allergic encephalomyelitis; ELISA, enzyme-linked immunosorbent assay; ESI-MS, electrospray ionisation mass spectrometry; FCC, flash column chromatography; Fmoc, (9*H*-fluoren-9-ylmethoxy)carbonyl; GBS, Guillan-Barré syndrome; Glc, β-D-glucopyranosyl; HATU, *N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-b]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HPLC, high performance liquid chromatography;

Ig, immunoglobulin; MBP, myelin basic protein; MeOH, methanol; MeONa, sodium methoxide; MOG, myelin oligodendrocyte glycoprotein; MRI, magnetic resonance imaging; MS, multiple sclerosis; Myel./Enc., parainfective and infective myelitis/encephalitis; NBS, normal bovine serum; NMM, *N*-methylmorpholine; PBS, phosphate-buffered saline; PLP, proteolipid protein; PNS, peripheral nervous system; TBTU, 1-[bis(dimethylamino)methylene]-1*H*-benzotriazolium 1-tetrafluoroborate 3-oxide; *t*Bu, *tert*-butyl; TFA, trifluoroacetic acid.

Amino acid symbols denote the L-configuration, unless otherwise indicated.

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10. ELISA were performed as follows: synthetic MOG peptides were dissolved at 0.01 mg/ml in 0.05 M carbonate buffer at pH 9.6 and coated 1μg/100 μl well onto 96-well microtitre plates (polystyrene ICN Flow, code 76-231-05). After incubation overnight at 4 °C, the plates were washed three times with PBS-T (0.01 M, containing 0.15 M NaCl and 0.05% Tween 20, pH 7.4) and blocked with 10% NBS (Sigma code S-7140) in PBS-T at room temperature for 1 h. The plates were washed three times and incubated with 100 μl/well of 1:100, 1:1000, 1:10000 dilution of human sera in 10% NBS-PBS-T for 16 h at 4 °C. With five washes between each step, we sequentially applied 100 μl/well of a dilution 1:500 in 10% NBS-PBS-T of the second anti-human IgG Ab, affinity purified and alkaline-phosphatase-conjugated (Sigma, code A 3312), and of a substrate solution containing 2 mg/ml *p*-nitrophenylphosphate in 10% diethanolamine buffer, 0.02% $MgCl_2$, pH = 9.6. The colorimetric reaction was stopped after 30 min adding 50μl/well of 1 M NaOH and the absorbance measured at 405 nm. The Ab levels in ELISA were expressed as titre, defined as the sample dilution reaching the blank absorbance (mean + 3 SD, corresponding in each plate to values below 0.2 absorbance units).
11. Sera were collected from patients affected by: MS (n = 5, autoimmune demyelinating disease); Myel./Enc. (n = 2, CNS inflammatory disease); ALS (n = 1, not-inflammatory CNS disease); GBS (n = 2, immune-mediated inflammatory PNS disease) and normal blood donors (n = 5).
12. All the peptides were synthesised by the continuous flow solid phase method on a semi-automatic apparatus (NovaSyn Gem Synthesiser) following the Fmoc/*t*Bu strategy. The resin for peptide acids, Fmoc-Leu-NovaSyn-TGA (0.20 mmol/g) and the Fmoc-protected amino acids were purchased from Novabiochem (Switzerland). Fmoc-protected amino acids were used in 2.5-fold excess and activated by HATU/NMM in DMF. HATU was purchased from PerSeptive Biosystems (USA). Deprotection reactions were accomplished with 20% piperidine in DMF. DMF was maintained over molecular sieves and stored

under nitrogen; piperidine was distilled from KOH. Acylation end points were determined by checking that the absorbance at 597 nm, due to the release of an anionic dye (acid Violet 17) from the cationic resin bound amino groups, did not change by more than 2 absorbance units over 10 min after a recirculation time of 30 min. Deprotection reactions were followed by monitoring at λ 365 nm the resulting dibenzofulvene-piperidine adduct. On completion of the synthesis the resin was washed with DCM, ether and dried *in vacuo*. Peptides were cleaved and the side-chains deprotected at room temperature by TFA/thioanisole/phenol/1,2-ethanedithiol (94 : 2 : 2 : 2). Deacetylation of the precursors of MOG2, monitored by HPLC, was achieved dissolving the crude material in dry MeOH and adding 0.1 M MeONa until pH 12. The mixture was stirred at room temperature for 2 h, neutralised with solid CO_2 and concentrated. Debenzoylation of the precursors MOG3 and MOG4, accomplished according the procedure above described for deacetylation at pH = 12, was complete in 1 h. Crude peptides were purified by semi-preparative HPLC on a Vydac column ODS 218TP1010 (250 × 10 mm). Analytical HPLC was performed on a Vydac ODS 218TP54 (250 × 4 mm), using the indicated gradient with the following eluants: A, 0.1% TFA in H_2O; B, 0.1% TFA in CH_3CN. HPLC-grade solvents were purchased from Carlo Erba (Italy). All other chemicals were commercially pure compounds and were used as received. Characterisation of the products was performed using analytical HPLC, ESI-MS spectrometry (Micromass Model VG Quattro apparatus) and amino acid analysis, that was carried out on a Biotronic LC 6001 apparatus after hydrolysis of peptide samples for 48 h at 110 °C in 6 M HCl containing 2.5% thioglycolic acid. Melting points were determined with a Büchi apparatus and are uncorrected. Final HPLC purity of the peptides was always > 98%. Yields of the purified peptides were calculated as percentage of the theoretical yield, based on the substitution level of the resin.

13. Amino acid analyses with theoretical values in brackets. **MOG1**: Ala 0.94 (1), Asn 0.89 (1), Arg 1.92 (2), Gly 1.88 (2), Glu 0.89 (1), His 0.95 (1), Leu 0.86 (1), Lys 0.98 (1), Met 0.93 (1), Phe 0.99 (1), Pro 1.82 (2), Ser 1.00 (1), Thr 0.88 (1), Trp 0.91 (1), Val 2.88 (3); **MOG2**: Ala 0.90 (1), Asn 0.96 (1), Arg 1.90 (2), Gly 1.94 (2), Glu 0.88 (1), His 0.98 (1), Leu 0.84 (1), Lys 0.95 (1), Met 0.89 (1), Phe 0.93 (1), Pro 1.86 (2), Ser 0.90 (1), Thr 0.88 (1), Trp 0.95 (1), Val 2.92 (3); **MOG3**: Ala 0.89 (1), Arg 1.90 (2), Gly 1.92 (2), Glu 0.87 (1), His 0.98 (1), Leu 0.96 (1), Lys 0.88 (1), Met 0.93 (1), Phe 0.93 (1), Pro 1.80 (2), Ser 2.00 (2), Thr 0.89 (1), Trp 0.96 (1), Val 2.86 (3); **MOG4**: Ala 0.92 (1), Arg 1.96 (2), Gly 1.93 (2), Glu 0.93 (1), His 0.97 (1), Hyp 0.84 (1), Leu 0.84 (1), Lys 0.95 (1), Met 0.93 (1), Phe 0.99 (1), Pro 1.82 (2), Ser 0.95 (1), Thr 0.86 (1), Trp 0.97 (1), Val 2.91 (3).
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