

Ferrocenyl Glycopeptides as Electrochemical Probes to Detect Autoantibodies in Multiple Sclerosis Patients' Sera

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ABSTRACT:

Glycopeptide analogues of CSF114(Glc), modified at N-terminus with new ferrocenyl carboxylic acid and a new ferrocenyl-thiophosphino amino acid, were used to implement a new electrochemical biosensor for autoantibody detection in multiple sclerosis. The ferrocenyl moiety of these "electrochemical probes" did not affect autoantibody recognition both in SP-ELISA and in inhibition experiments. By electrochemical monitoring the interactions of the modified peptides Fc-CSF114(Glc) and 4-FcPhP(S)Abu-CSF114(Glc) with the autoantibodies, we demonstrated that autoantibodies could be detected with a sensitivity comparable to ELISA method. The new electrochemical probes can be proposed to characterize autoantibodies as biomarkers of multiple sclerosis by a simple, rapid, and reproducible cyclic voltammetry-based

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INTRODUCTION

Autoimmune disorders have a high social impact, affecting mainly young adults and in particular women. Clinical evaluation of autoimmune diseases can require several years before it can be definitively ascertained. Both genetic predisposition and environmental factors could play a synergistic role in the autoimmune diseases. Most of them are characterized by relapsing-remitting forms and adequate therapies should be used early in the disease course and during flares, to prevent chronic damages. Thus, reliable assays are necessary not only for an early diagnosis but also for monitoring the disease activity by evaluating the antibodies, as specific biomarkers, for diagnosis, follow-up, and prevention of the autoimmune disease.^{1,2} The autoantibody fluctuation with disease exacerbations or remissions, makes their detection extremely valuable in the follow up of patients.³ Therefore, quantitative and qualitative measurements of autoantibodies are crucial in the

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management of autoimmune disorders, particularly in the development and clinical evaluation of personalized therapeutic treatments.

To help clinicians to follow up patients by a simple blood test detecting autoantibodies two different problems have to be solved, such as: (1) selection of the antigen recognizing specific autoantibodies in a statistically significant number of patients (sensitivity) as compared to other autoimmune diseases and normal controls; (2) selection of the diagnostic technique to detect autoantibodies [“enzyme-linked immunosorbent assay” (ELISA), radioimmunoassay, immunofluorescence, SPR, etc.].

Till now, identification of autoantibodies is achieved using native protein antigens in ELISA. Unfortunately, in autoimmune diseases only very low specific antibodies were detected in serum, possibly because native protein antigens used in the assays contain more specific epitopes for different autoantibodies. Moreover, the use of recombinant protein antigens does not allow replication of possible aberrant modifications (sugars, lipids, citrullines, etc.), involved in triggering an autoantibody response. In fact, it is generally accepted that these modifications can alter the function and immunogenicity of protein/peptide antigens and growing evidences indicate that post-translational modifications, either native or aberrant, may play a fundamental role for specific autoantibody recognition in autoimmune diseases.⁴ These observations account, at least in part, for the limited success get in the discovery of biomarkers for autoimmune disorders using proteomic analysis and/or protein microarrays. For that reason we have decided to develop and optimize new synthetic peptides as antigenic probes for fishing out autoantibodies from biological fluids as biomarkers using an innovative “chemical reverse approach.” “Reverse” because the screening of the synthetic antigenic probe is guided by autoantibodies circulating in blood and “chemical” because autoantibody recognition drives selection and optimization of the “chemical” structure by defined peptide libraries.

The screening of focused libraries of modified peptides has to lead to the optimized peptide antigen containing the minimal epitope with the correct modification to detect at the best autoantibodies specific of the autoimmune disease under investigation.⁵ As a proof-of-concept, in the case of rheumatoid arthritis (RA) several aberrant modifications of proteins (such as Arg deimination and/or methylation) have been associated with pathogenesis and lead to different modified autoantigens used in simple ELISA.⁴ A key step in setting up the commercially available ELISA for RA was represented by identification of deiminated sequences of filaggrin that are recognized by a high percentage of RA sera.^{6,7} Sensitivity of the assay was increased modifying the peptide structure to optimally expose the citrulline moiety. In fact, a

cyclic citrullinated peptide allows detection of antibodies in up to 70% of RA patients.^{8,9} This ELISA is now considered a gold standard for RA diagnosis and follow up.

In the case of multiple sclerosis (MS), one of the most known inflammatory neurological diseases of the central nervous system (CNS), patients accumulate myelin lesions, but also axonal loss, which lead to permanent neurological dysfunctions. Magnetic resonance imaging (MRI) is up to now the most reliable technique to help clinicians not only in diagnosis, but also in prognosis because no standard simple immunoassays are available. However, it is evident that MRI cannot be considered a routine technique and that efficient autoantibodies detection is a useful signal of disease progression when the clinical symptoms are not still visible to guide a targeted MRI checkup.¹⁰

In previous studies, we developed for the first time the glycopeptide CSF114(Glc), a structure-based designed glucosylated peptide as the first MS Antigenic Probe (MSAP),^{11,12} accurately measuring high affinity autoantibodies in sera of a statistically significant patients' population.¹³ The glycopeptide is characterized by a β -hairpin structure bearing the minimal epitope (a β -D-glucopyranosyl moiety linked to an Asn residue on the tip of the turn probably reproducing an aberrant *N*-glucosylation of myelin) fundamental for autoantibody recognition.^{14–16}

ELISA diagnostic/prognostic test MSPepKit,¹⁷ based on CSF114(Glc), has been developed to recognize specific autoantibodies in MS patients' sera and follow up disease activity.¹⁸ ELISA offers advantages, such as allowing simultaneous analyses of a large number of samples. To avoid nonreproducible or noninterpretable results due to operator-dependent procedures, industry requires not only a fast, but also a sensitive and more consistent technique, in particular for quantitative determination of autoantibodies.

Electrochemistry, as detection technique of biological and clinical assays, can shorten the time of the analyses and increase the reliability of the assays.

RESULTS AND DISCUSSION

To implement a new electrochemical technique for autoantibody recognition in MS, we used CSF114(Glc) analogues properly modified at N-terminus with new ferrocenyl derivatives (see Figure 1) as “electrochemical probes” to be used in cyclic voltammetry measurements in solution and/or grafting peptides on a gold electrode.

Availability of a large variety of ferrocenyl derivatives and their favorable electrochemical properties (undergoing a reversible oxidation in aqueous solution) have made ferrocene and its derivatives very trendy molecules for biological applications and for conjugation with biomolecules.¹⁹ Therefore, ferrocenyl derivatives are a new class of linkers useful for

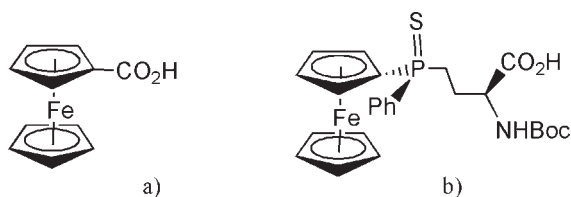


FIGURE 1 Structure of ferrocenyl derivatives used for SPPS. a) Ferrocenyl carboxylic acid [Fc-COOH] and b) (S,S,S) -(+)-2-(*tert*-butoxycarbonylamino)-4-(ferrocenylphenylthiophosphino)butanoic acid [4-FcPhP(S)Abu].

electrochemical and optical biodetections. In this context, we selected ferrocenyl carboxylic acid and a new ferrocenyl-thiophosphine derivative of α -aminobutyric acid (see Figure 1) as biosensors.

The electrochemical properties of ferrocene, coupled to thiophosphine ability to build simple monolayers on gold surfaces, allow peptides anchoring on the working electrode used for detection. The possibility of grafting the synthetic probe directly on the electrode surface will enable to obtain an innovative strategy to develop new techniques for antibody detection opening strategies in the analysis of antibody profiles in MS patients' sera, possibly improving detection of a panel of antibodies as specific biomarkers for different forms of the disease.

Labeled Peptide Synthesis

A collection of labeled peptides was prepared by solid phase peptide synthesis (SPPS) using the Fmoc/*t*Bu strategy (Table I). The novel ferrocenyl peptides were synthesized modifying CSF114(Glc) sequence TPRVERN(Glc)GHSVFLAPYGWMVK and the corresponding unglucosylated one TPRVERNHSVFLAPYGWMVK, at the N-terminus with the designed ferrocenyl derivatives (see Figure 1). The amino group of 4-FcPhP(S)Abu was Boc protected and the carboxylic acid of both derivatives was free to be used directly in SPPS.

Only few studies are reported on the solid-phase synthesis of organometallic derivatives of peptides. In fact, decomposi-

tion of organometallic peptides and secondary products of SPPS usually affects final yields.²⁰ Because microwave technology is proposed as a valid support for enhancement of efficiency of coupling reactions in SPPS, we applied this strategy to synthesize the peptide sequences using a microwave assisted automatic solid-phase peptide synthesizer. Microwave technology decreases chain aggregation during the syntheses, improves the coupling rates, and prevents side reactions in difficult peptide sequences.²¹

Ferrocenyl carboxylic acid and the Boc-protected organometallic amino acid 4-FcPhP(S)Abu (see Figure 1) were introduced at the N-terminus of the resin-bound glycopeptide I and peptide I' to obtain the peptide collection reported in Table I.

The metallocene moieties were demonstrated to be stable during Fmoc-deprotection, cleavage, and in the deacetylation conditions (pH 12) requested for deprotection of the hydroxyl groups of the sugar. However, the final ferrocenyl peptides could be safely obtained when phenol rather than water was used as scavenger in the final cleavage mixture for resin and amino acid side chains deprotection.²⁰

All the compounds were analyzed by UPLC-ESIMS and HPLC-ESIMS and purified by RP-HPLC (>95%) to be used for autoantibody detection.

Immunoenzymatic Assays

The glycopeptide CSF114(Glc) (I), designed as a type I' β -turn around the minimal epitope Asn(Glc), guarantees an optimal exposure of the epitope for antibody recognition in the solid-phase conditions of the ELISA plate on sera of MS patients. In fact, the CSF114(Glc)-based ELISA allows to recognize both IgM and IgG in sera of 30% of MS patients.¹³

We evaluated, by ELISA, serum antibodies (IgM and IgG class) to the new organometallic peptides and glycopeptides (Table I) in a group of MS patients and compared the results with normal blood donors' sera (NBDs). IgM and IgG

Table I Analytical Data from Ultra Performance Liquid Chromatography (UPLC)

Peptide Name	R_t (min)	RP-HPLC Preparative Gradients of B	Calculated Mass	Observed Mass $[M+2H]^{2+}$
CSF114(Glc) (I)	2.07 ^a	28–30% in 30 min	2606.3	1303.6
Fc-CSF114(Glc) (II)	1.872 ^b	35–70% in 20 min	2820.2	1411.2
4-FcPhP(S)Abu-CSF114(Glc) (III)	1.833 ^b	25–50% in 20 min	3015.5	1508.3
CSF114 (I')	1.96 ^a	28–30% in 30 min	2444.2	1222.6
Fc-CSF114 (II')	1.813 ^b	25–50% in 20 min	2655.3	1329.8
4-FcPhP(S)Abu-CSF114 (III')	1.863 ^b	35–70% in 20 min	2853.5	1427.4

Gradients at 0.45 ml min^{-1} . (Solvent system, A: 0.1% TFA in H_2O , B: 0.1% TFA in CH_3CN).

^a 10–60% B in 3.5 min.

^b 20–70% B in 3.5 min.

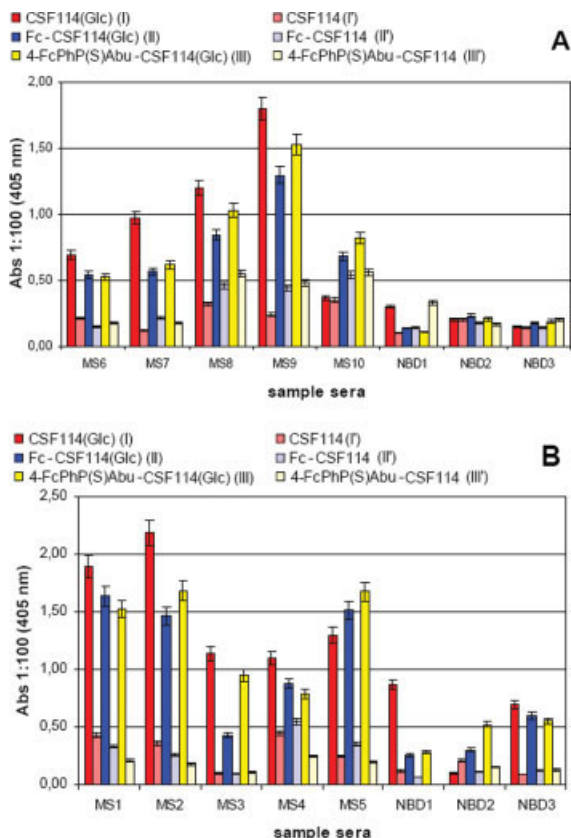


FIGURE 2 Autoantibody recognition in MS patients' sera and NBDs sera. IgM (A) and IgG (B) classes versus the ferrocenyl glycopeptides (II–III) and the corresponding unglycosylated ones (I'–III') compared to the glycopeptide CSF114(Glc) (I). Data are reported as absorbance at 405 nm of sera diluted 1:100.

responses were detected using as secondary antibodies anti-human IgMs and anti-human IgGs conjugated to alkaline phosphatase.

We compared the antibody recognition to CSF114(Glc) versus the ferrocenyl glycopeptides II and III and the corresponding unglycosylated sequences I'–III' in a first group of patients' sera affected by clinically definite MS using SP-ELISA. It is accepted that the glycopeptide I detects specific antibodies in MS patients' sera.¹¹ In fact, as we observe in Figure 2, the glycopeptide I presents the highest antibody recognition, but the new ferrocenyl glycopeptides II–III are always able to detect antibodies showing only a relative lower biological recognition. As expected unglycosylated peptides I'–III' are always inactive.

Considering that SP-ELISA reflects essentially the relative affinity, which depends on the exposure of the minimal epitope in the solid phase conditions of the assay, we also investigated the absolute antibody affinity in a competitive ELISA based on inhibition of autoantibodies in solution. In a set of MS positive sera, ferrocenyl glycopeptides II–III inhibited

binding of autoantibodies to I, giving rise to similar inhibition curves. The data of a representative serum (see Figure 3) show that the two modified glycopeptides II and III exhibited equivalent affinity in competitive ELISA, despite the differences of apparent affinity detected in SP-ELISA (see Figure 2). These findings indicated that the new ferrocenyl glycopeptides share an epitope similar to the one presented by I, and the presence of ferrocenyl and/or thiophosphine moiety at the N-terminus does not influence antibody recognition.

Therefore the glycopeptide CSF114(Glc), modified with the ferrocenyl derivatives, is still able to detect and inhibit autoantibodies in MS patients' sera and it cannot detect any antibody titer in NBDs' sera.

Electrochemical Measurements

Grafted Antigen on Gold. The glucosylated ferrocenyl peptides 4-FcPhP(S)Abu-CSF114(Glc) (III) and the unglycosylated one 4-FcPhP(S)Abu-CSF114 (III') were adsorbed on gold surface to form a self-assembled monolayer (SAM) *via*

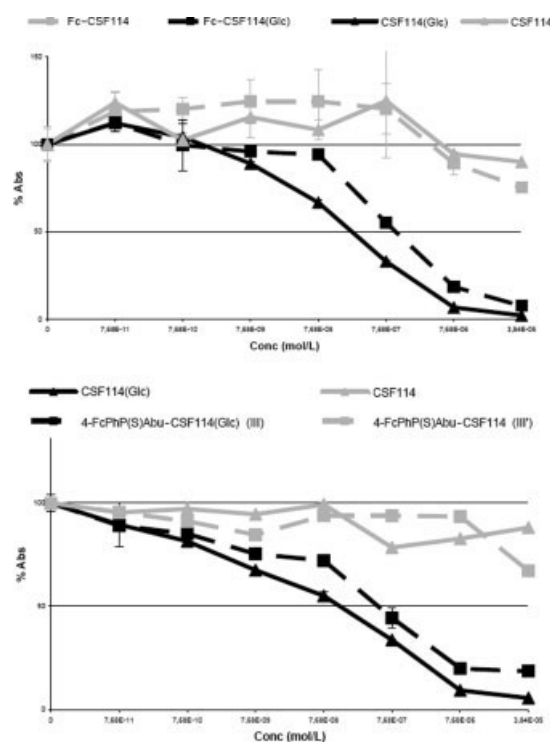


FIGURE 3 Inhibition curves of anti-CSF114(Glc) IgG Abs with ferrocenyl glycopeptides II–III and with unglycosylated peptides I'–III', in comparison with I in a competitive ELISA. The results are expressed as the percentage of absorbance of a representative MS serum (ordinate axis).

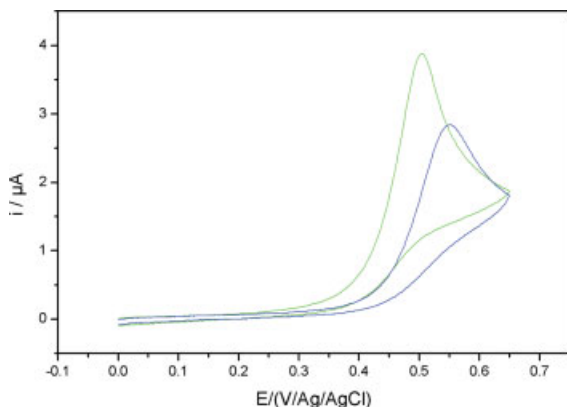


FIGURE 4 Cyclic voltammetry of Au-III in a tween solution containing LiClO_4 0.1M and $\text{Fe}(\text{CN})_6^{4-}$ 9×10^{-4} M (green curve) without antibodies and with a solution of purified anti-CSF114(Glc) antibodies at a final dilution of 1:1000 (blue curve). Scan rate 50 mV s^{-1} .

the sulfur atom. A mixture of 11-mercaptoundecanoic acid and decanethiol was used to block the uncovered surface. The modified electrodes are denoted Au-III for the electrode grafted with III and Au-III' for the electrode grafted with III'. The electrochemical response of the ferrocenyl group was performed by cyclic voltammetry. $\text{Fe}(\text{CN})_6^{4-}$ was used as catalyst to increase the electrochemical current.²² Figure 4 shows the electrochemical response of Au-III in a tween solution of $\text{Fe}(\text{CN})_6^{4-}$. When a solution of purified anti-CSF114(Glc) antibodies was added, the electrochemical response was shifted towards more positive potentials. The peak potential appeared 46 mV more positive than that of the same solution without antibodies.

When the same experiment was repeated with Au-III', i.e. with the electrode modified by the unglucosylated peptide III', which is not able to recognize autoantibodies in ELISA, the results were completely different (see Figure 5). The electrochemical response of Au-III' did not change significantly after the addition of a solution of purified anti-CSF114(Glc) antibodies. The peak potential was even shifted by few millivolts toward more negative potentials.

Antigen in Solution. The detection of interactions between autoantibodies and the ferrocenyl peptides, Fc-CSF114(Glc) (II), i.e. without P=S anchor, was realized in solution. The gold electrode, properly modified with 11-mercaptoundecanoic acid to avoid nonspecific interactions with the biological medium, was introduced in a 0.05% tween 20 solution containing both 0.1 M LiClO_4 and 9×10^{-4} M $\text{Fe}(\text{CN})_6^{4-}$ and the ferrocenyl glycopeptide II. Figure 6 shows the cyclic voltammograms before and after addition of anti-CSF114(Glc) antibodies.

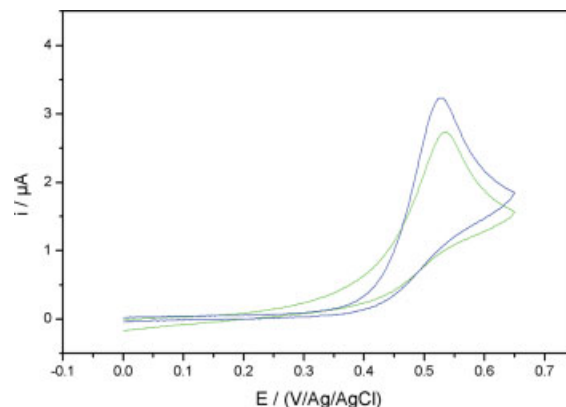


FIGURE 5 Cyclic voltammetry of Au-III' in a tween solution containing LiClO_4 0.1M and $\text{Fe}(\text{CN})_6^{4-}$ 9×10^{-4} M (green curve) without antibodies and with a solution of purified anti-CSF114(Glc) antibodies at a final dilution of 1:1000 (blue curve). Scan rate: 50 mV s^{-1} .

In the case of the glucosylated ferrocenyl peptide II, the peak potential was shifted of 34 mV towards positive values by addition of anti-CSF114(Glc) antibodies. However, in this case, a study of the nature of the protective monolayer should be investigated to confirm the interest of the glycopeptide II for autoantibody detection.

The results are summarized in Figure 7. The glucosylated biosensor Au-III and the glucosylated ferrocenyl peptide II in solution shift of more than 30 mV towards positive values by addition of anti-CSF114(Glc) antibodies. This is indicative of detection of antigen–antibody interaction, and therefore of the presence of autoantibodies in the tested MS patients' sera. The negative control experiments performed with the unglucosylated biosensors showed a very small potential

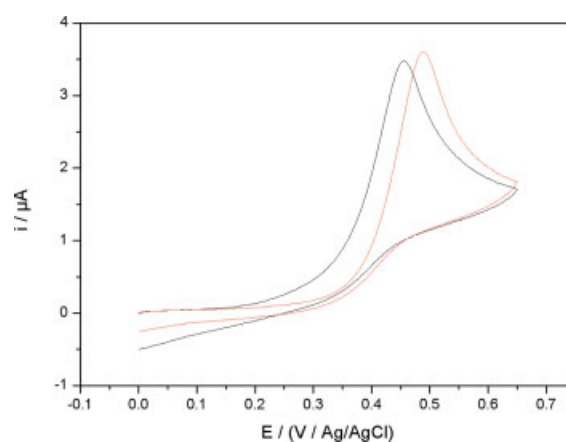


FIGURE 6 Cyclic voltammetry of II, 1.77×10^{-4} M in a tween solution containing LiClO_4 0.1M and $\text{Fe}(\text{CN})_6^{4-}$ 9×10^{-4} M (black curve) without antibodies and (red curve) with a solution of purified anti-CSF114(Glc) antibodies at a final dilution of 1:1000. Scan rate: 50 mV s^{-1} .

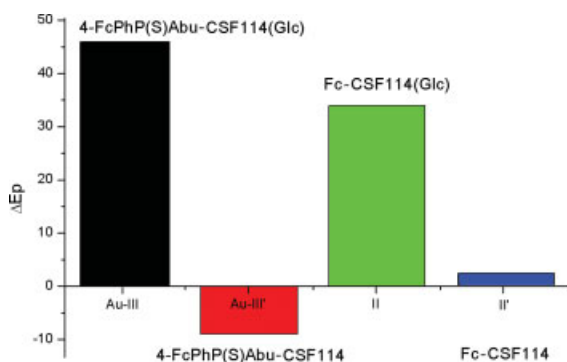


FIGURE 7 Electrochemical immunoassay: potential difference between the electrochemical response with and without purified anti-CSF114(Glc) antibodies at a final dilution of 1:1000 for (black) the biosensor Au-III, (red) its negative control Au-III', (green) the ferrocenyl glycopeptide II in solution and (blue) its negative control II' in solution.

shift, positive in the case of the ferrocenyl peptide II' and in the opposite direction for Au-III'.

CONCLUSIONS

In conclusion, the new ferrocenyl peptides containing the unnatural amino acid 4-FcPhP(S)Abu are able to detect specific anti-CSF114(Glc) antibodies by electrochemical technique. Moreover, thanks to the presence of thiophosphine, the modified glycopeptide 4-FcPhP(S)Abu-CSF114(Glc) (III) is able to build simple monolayers on gold surfaces and it can be useful for antibody detection and quantitative determinations. The possibility of grafting these new ferrocenyl glycopeptides, as synthetic antigenic probes, directly on the electrode surface (as a valid alternative analytical method to detect autoantibodies in sera of MS patients) is currently under investigation in our laboratories.

In this article, we demonstrated the possibility of detecting isolated antibodies by Cyclic Voltammetry in samples containing 1:1000 diluted antibodies. This detection limit is in any case lower compared to the one set up in our validated ELISA on sera.^{11,12} Therefore, we are confident that this new voltammetry-based technique could be useful in detecting antibodies in sera of multiple sclerosis patients.

EXPERIMENTAL PROCEDURES

Materials

Fmoc-protected amino acids and Fmoc-Lys(Boc)-Wang resin were obtained from Novabiochem AG (Laufelfingen, Switzerland). 2-(1*H*-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) was from Iris-Biotech. Peptide-synthesis grade *N,N*-dimethylformamide (DMF) was from Scharlau (Barcelona, Spain).

Trifluoroacetic acid (TFA), dichloromethane (DCM), piperidine, *N*-methylmorpholine (NMM), *N,N*-diisopropylethylamine (DIPEA), 11-mercaptoundecanoic acid, decanethiol, and ferrocene carboxylic acid (FcCOOH) were purchased from Aldrich. 1-Hydroxy-7-azabenzotriazole (HOAt) was from PerSeptive Biosystems. HPLC-grade acetonitrile (MeCN) was purchased from Carlo Erba (Italy).

Synthesis of (*S_p*,*S*)-(+)-2-(*tert*-butoxycarbonylamino)-4-(ferrocenylphenylthiophosphino)butanoic acid

The ferrocenyl thiophosphine amino acid [4-FcPhP(S)Abu] was synthesized in 48% yield,²³ by reaction of (*S*)-ferrocenylphenylphosphine borane with the benzyl (*S*)-(-)-2-[bis(*tert*-butoxycarbonyl)amino]-4-iodobutanoate, (previously prepared according a modified procedure),²⁴ then subsequent sulfuration and deprotection to afford the free carboxylic acid group.

(*S_p*,*S*)-(+)-2-(*tert*-butoxycarbonylamino)-4-(ferrocenylphenylthiophosphino) butanoic acid:¹⁹ orange powder; $\alpha_D = +49$ ($c = 0.6$, CHCl₃); ¹H NMR (CDCl₃) δ 1.35 (s, 9H, t-Bu), 1.79 (m, 1H, CHH), 2.26–2.28 (m, 3H, CH₂, CHH), 4.12 (s, 5H, Cp), 4.27–4.55 (m, 5H, CpP, CHN), 5.15 (sl, 0.5H, NH), 6.67 (sl, 0.5H, NH), 7.41 (m, 3H, Ph), 7.82 (m, 2H, Ph); ³¹P NMR (CDCl₃) δ +42.6; HRMS (ESI, *m/z*) Calcd for C₂₅H₃₀FeNO₄PS: 527.0977 (*M*⁺); observed: 527.0931. Anal. Calcd for C₂₅H₃₀FeNO₄PS: C, 56.94; H 5.73; N 2.66. Found: C, 56.26; H 5.94, N, 2.52.

Peptide Synthesis

Solid-phase peptide synthesis (SPPS) was performed by microwave assisted automatic peptide synthesizer LibertyTM Microwave Peptide Synthesizer (CEM Corporation, Matthews, NC), an additional module of DiscoverTM (CEM Corporation, Matthews, NC) that combines microwave energy at 2450 MHz following the fluorenylmethoxycarbonyl (Fmoc)/*tert*-butyl (*t*Bu) strategy. CSF114 and CSF114(Glc) were prepared starting from a Fmoc-Lys(Boc)-Wang resin (0.67 mmol/g). Fmoc deprotections were performed with a 20% piperidine in DMF solution. Fmoc amino acids were stored as 0.3M DMF solutions. Coupling reagent was predissolved in DMF (0.3M solution). Coupling reactions were performed with 2.5 equiv. of TBTU in DMF (0.25M), 2.5 equiv. of amino acids in DMF (0.1M), and 3.5 equiv. of DIPEA in NMP solution (0.7M). Glucosyl moiety of glycosylated peptide I was introduced as the building-block Fmoc-L-Asn[GlcOAc4]-OH (2.5 equiv.) which was synthesized as previously reported.¹⁶ Deprotection and coupling reactions were performed with microwave energy and nitrogen mixing. Microwave cycle was characterized by two deprotection steps (30 s, 180 s). All microwave coupling reactions were of 300 s at 75°C.

Ferrocenyl carboxylic acid (Fc-COOH) (2.0 equiv.) was coupled with TBTU (2 equiv.), HOBt (2 equiv.), and NMM (3 equiv.) in the *N*-terminus of CSF114(Glc) and CSF114 sequences, respectively, for 1.5 h on a manual batch synthesizer (PLS 4 × 4, Advanced ChemTech) using Teflon reactors (10 ml) obtaining peptides II and II'. (*S_p*,*S*)-(+)-2-(*tert*-butoxycarbonylamino)-4-(ferrocenylphenylthiophosphino)butanoic acid was coupled with TBTU (2 equiv.), HOBt (2 equiv.), and NMM (3 equiv.) in the CSF114(Glc) and CSF114 sequences respectively for 1.5 h on a manual batch synthesizer obtaining peptides III and III'.

Peptide cleavages from the resin and deprotection of the amino acids side chains were carried out with 1 ml/100 mg of resin of TFA/

anisole/1,2-ethanedithiol/phenol/H₂O solution (96:1:1:1 v/v/v/v). The cleavage was maintained for 3 h at room temperature. The resins were washed with TFA and the filtrates partially evaporated. The crude products were precipitated with diethyl ether, collected by centrifugation, dissolved in H₂O and lyophilized with an Edwards apparatus, model Modulyo.

Deacetylation of sugar moieties linked to the peptides I–III was performed with a 0.1 M methanolic NaOMe solution until pH 12 to a solution of the lyophilized peptides in dry MeOH (1 ml/100 mg of resin). After 3 h the reaction was quenched by adding concentrated HCl until pH 7, the solvent was evaporated under vacuum and the residue lyophilized.

All peptides were purified by semipreparative RP-HPLC Waters mod. 600 with Jupiter C18 (10 μ m, 250 mm $\times$ 10 mm), at 4 ml min⁻¹ using methods described in Table I and the purity of the peptides was analyzed using a Waters Alliance (model 2695) with Jupiter Phenomenex column (5 μ m, C18, 200 Å, 250 mm $\times$ 4.6 mm of diameter) at 1 ml min⁻¹ of a mixture of eluents: (A) 0.1% TFA in H₂O (MilliQ) and (B) 0.1% TFA in CH₃CN, λ = 254 nm.

Characterization of the products were performed by Ultra Performance Liquid Chromatography using an ACQUITY UPLC (Waters Corporation, Milford, Massachusetts) coupled to a single quadrupole ESI-MS (Micromass ZQ) using a 2.1 mm $\times$ 50 mm 1.7 μ m ACQUITY BEH C18 at 30°C, with a flow rate of 0.45 ml min⁻¹.

Immunological Assays

Serum was obtained for diagnostic purposes from patients and healthy blood donors who had given their informed consent, and stored at -20°C until use.

Antibody responses were determined in SP-ELISA. Ninety-six-well activated polystyrene ELISA plates (NUNC Maxisorb SIGMA) were coated with 1 μ g per 100 μ l of peptides per well or glycopeptides in pure carbonate buffer 0.05 M (pH 9.6) and incubated at 4°C overnight. After five washes with saline containing 0.05% Tween 20, nonspecific binding sites were blocked by fetal calf serum, 10% in saline Tween 20 (100 μ l per well) at room temperature for 60 min. Sera diluted from 1:100 to 1:100,000 were applied at 4°C overnight in saline/Tween 20/10% FCS. After five washes, we have added 100 μ l of alkaline phosphatase conjugated anti-human IgM (diluted 1:200 in saline/Tween 20/FCS) or IgG (diluted 1:8000 in saline/Tween 20/FCS) (Sigma) to each well. After 3 h at room temperature incubation and five washes, 100 μ l of substrate solution consisting of 1 mg/ml *p*-nitrophenyl phosphate (Sigma) in 10% diethanolamine buffer was applied. After 30 min, the reaction was stopped with 1 M NaOH (50 μ l), and the absorbance was read in a multi-channel ELISA reader (Tecan Sunrise) at 405 nm. ELISA plates, coating conditions, reagent dilutions, buffers, and incubation times were tested in preliminary experiments.¹¹ The antibody levels are expressed as absorbance in arbitrary units at 405 nm (sample dilution 1:100).

Antibody affinity was measured by following the methods reported.²⁵ The semi-saturating sera dilution (1:600) was calculated from the preliminary titration curves (absorbance, 0.7). At this dilution, Abs were preincubated with increasing synthetic peptide antigen concentration (0; 7.68 E-11; 7.68 E-10; 7.68 E-09; 7.68 E-08; 7.68 E-07; 7.68 E-06; 3.84 E-05) for 1 h at room temperature. Unblocked Abs was revealed by ELISA, and the antigenic probe concentration-absorbance relationship was presented graphically.

Electrochemical Apparatus

In a cyclic voltammetry (CV) experiment, the potentiostat applied a potential ramp to the working electrode to gradually change the potential and then reversed the scan, returning to the initial potential at a constant scan rate.

The electrochemical instrumentation includes an EG&G 283 potentiostat connected to a PC and the collected data were analyzed using a Princeton Applied Research Software, Power Suite.

A special electrochemical cell was used to handle only few microliters. The auxiliary electrode is a platinum wire and the reference is an Ag/AgCl electrode separated from the solution by a vycor tip. The active working electrode is a 1.6 mm diameter gold electrode (BAS) protected by 11-mercaptopundecanoic acid SAM for the ferrocenyl glycopeptide II. In the case of III and III', a mixture of 11-mercaptopundecanoic acid and decanethiol was used after immobilization of the ferrocenyl glycopeptides.

The electrochemical measurements have been realized in a 0.05% tween 20 solution containing LiClO₄ 0.1 M and potassium ferrocyanide (K₄Fe(CN)₆, Aldrich), 9×10^{-4} M at room temperature. The concentration of Fc-CSF114(Glc) is 1.77×10^{-4} M. Specific autoantibodies were diluted at 1:1000. All solutions were deoxygenated prior to experiments.

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