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RESEARCH ARTICLE

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Glucopeptides derived from myelin-relevant proteins and hyperglucosylated nontypeable *Haemophilus influenzae* bacterial adhesin cross-react with multiple sclerosis specific antibodies: A step forward in the identification of native autoantigens in multiple sclerosis

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Multiple sclerosis (MS) is an inflammatory and autoimmune disorder, in which an antibody-mediated demyelination mechanism plays a critical role. We prepared two glucosylated peptides derived from the human myelin proteins, that is, oligodendrocyte-myelin glycoprotein (OMGp) and reticulon-4 receptor (RTN4R), selected by a bioinformatic approach for their conformational homology with CSF114(Glc), a designed β-turn antigenic probe derived from myelin oligodendrocyte glycoprotein (MOG), a glycoprotein present in the CNS. This synthetic antigen is specifically recognized by antibodies in sera of MS patients. We report herein the antigenic properties of these peptides, showing, on the one hand, that MS patient antibodies recognize the two glucosylated peptides and, on the other hand, that these antibodies cross-react with CSF114(Glc) and with the previously described hyperglucosylated nontypeable *Haemophilus influenzae* bacterial adhesin protein HMW1ct(Glc). These observations point to an immunological association between human and bacterial protein antigens, underpinning the hypothesis that molecular mimicry triggers the breakdown of self-tolerance in MS and suggesting that RTN4R and OMGp can be considered as autoantigens.

KEYWORDS
autoantibodies, autoantigens, glucopeptides, molecular mimicry, multiple sclerosis

Abbreviations: Abs, antibodies; ACN, acetonitrile; CD, circular dichroism; CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; ELISA, enzyme-linked immunosorbent assay; FAN, factor associated with neutral sphingomyelinase activation; FBS, fetal bovine serum; Fmoc, fluorenylmethyloxycarbonyl; Glc, β-D-glucopyranosyl; HMW1ct, *C*-terminal adhesin of nontypeable *Haemophilus influenzae*; IC_{50}, half maximal inhibitory concentration; IgG, immunoglobulin G; LRR, leucine-rich-repeat; MAG, myelin-associated glycoprotein; MS, multiple sclerosis; MW, microwave; NTHi, nontypeable *Haemophilus influenzae*; OMG, oligodendrocyte-myelin glycoprotein; PBS, phosphate-buffered saline; RTN4R, reticulon-4 receptor; ESI-MS, electrospray ionization-mass spectrometry; SDS, sodium dodecylsulfate; SPPS, solid-phase peptide synthesis; *t*Bu, *tert*-butyl; TNF, tumor necrosis factor.

This paper is dedicated to Prof. Ulf Diederichsen.

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1 | INTRODUCTION

Multiple sclerosis (MS) is a complex, heterogeneous, inflammatory disorder characterized by a loss of myelin sheath surrounding the nerve axons in the central nervous system (CNS). MS is considered an autoimmune disease, but its etiology is still largely unknown, although it is established that complex interactions between environmental factors and multiple genetic factors are involved.[1,2] At the immunological level, the hypothesis that an antibody-mediated demyelination mechanism might contribute to the immunopathology of MS is increasingly recognized.[3–5] Our findings contributed support to this hypothesis showing that sera of an MS patient subpopulation present antibodies specific to a structure-based designed β-turn glucopeptide, termed CSF114(Glc) that is characterized by the presence of an unusual *N*-glucosylation on asparagine (N-Glc).[6,7]

Subsequently, looking for a putative native autoantigen mimicked by CSF114(Glc), we implemented a bioinformatic approach to screen human myelin proteins for homologies to CSF114(Glc). Because the recognition of the synthetic antigen by MS-specific autoantibodies appears to be dependent on the peptide sequence and on its predominant β-turn conformation, we performed both a sequence and a 3D alignment study.[8] This analysis yielded three sequences featuring either sequence or conformational homology with the designed glucopeptide CSF114(Glc), belonging to three relevant myelin proteins: factor associated with neutral sphingomyelinase activation protein (FAN), oligodendrocyte-myelin glycoprotein (OMGp), and reticulon-4 receptor (RTN4R). FAN (UniProtKB/Swiss-Prot accession number: Q92636) is an ubiquitous protein that plays a key role in the regulation of ceramide production through interaction with neutral sphingomyelinase (N-Smase) in response to various stimuli, including tumor necrosis factor (TNF).[9] OMGp (UniProtKB/Swiss-Prot accession number: P23515) is a CNS protein expressed on the surface of oligodendrocytes that has multiple functions, including regulating axonal regrowth and contributing to the formation and maintenance of myelin sheath.[10,11] Recent studies have also shown the presence of anti-OMGp antibodies in some sera from MS patients and that OMGp-specific T cells are able to induce a new type of EAE animal model characterized by meningitis.[12] RTN4R (UniProtKB/Swiss-Prot accession number: Q9BZR6), also known as Nogo receptor, is also a CNS protein that is very similar in structure to OMGp, mainly expressed in the grey matter of the brain, belonging to the leucine-rich-repeat (LRR) protein family with a globular structure.[13,14] It regulates axonal regrowth and plasticity after injury by interacting with other proteins, including OMGp and myelin-associated glycoprotein (MAG).[15,16] In addition, anti-RTN4R antibodies are able to block MAG inhibition of neuronal growth.[16] In our previous study,[8] the three selected sequences, modified by with a β-D-glucopyranosyl moiety on an Asn residue and tested in competitive ELISA on MS patient sera, were found to cross-react with anti-CSF114(Glc) antibodies, thus demonstrating a mimicry between the designed antigenic probe and myelin proteins relevant in MS.

Finally, considering that the human glycoproteome repertoire does not include the simple *N*-glucosylation of asparagine, that is, the Asn(Glc) moiety, and taking into account that the progression of MS is also linked to exogenous infectious agents expressing antigenic molecules, which may mimic the structure and/or conformation of endogenous mammalian extracellular membrane embedded (glyco)proteins, we turned our attention to bacterial proteins. Accordingly, we were able to demonstrate that a cell-surface adhesin protein of nontypeable *Haemophilus influenzae* (NTHi) termed HMW1 and expressing a large number of *N*-glucosylations is preferentially recognized by antibodies from sera of an MS patient subpopulation cross-reacting with CSF114(Glc).[17] This was the first example of an *N*-glucosylated native antigen that can be considered a relevant candidate for triggering pathogenic antibodies in MS, due to a possible molecular mimicry between self-molecules and an exogenous (bacterial) antigen.

Based on these evidences, the aim of the present study is to verify the possible cross-reactivity of MS patient antibodies recognizing the three (gluco)peptides selected through to the bioinformatic approach, that is, FAN, OMGp, and RTN4R, and the recombinant hyperglucosylated C-terminal fragment of NTHi, residues 1205–1536, that is, HMW1ct(Glc). Confirming the structural correspondence between an exogenous protein and a physiological one is the basis of the hypothesis that molecular mimicry triggers the breakdown of self-tolerance in MS.

2 | MATERIALS AND METHODS

2.1 | Reagents

All Fmoc-protected amino acids, *N*,*N*′-diisopropylcarbodiimide (DIC), and oxyma (ethyl cyanohydroxyiminoacetate) were purchased from Iris Biotech GmbH (Marktredwitz, Germany). Tentagel® S RAM resin was purchased from Rapp Polymere (Tuebingen, Germany). Peptide-synthesis grade *N*,*N*-dimethylformamide (DMF) and acetonitrile (ACN) were purchased from Carlo Erba (Milano, Italy). Dichloromethane (DCM), trifluoroacetic acid (TFA), triisopropylsilane (TIS), and piperidine were purchased from Sigma-Aldrich (Milano, Italy). Tris(hydroxymethyl) aminomethane hydrochloride (TRIS·HCl) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchased from VWR (Milano, Italy). Sodium dodecyl sulfate (SDS), guanidine hydrochloride, imidazole, glycerol, and Triton-X100 were purchased from Sigma-Aldrich (Milano, Italy). Fmoc-L-Asn[β-D-Glc(OAc)$_4$]-OH were synthesized as previously described.[18]

2.2 | Recombinant protein production: Expression and purification of adhesin proteins

The glucosylated HMW1ct(Glc) antigen, I(Glc), was obtained as previously described.[17] After cell lysis performed using ice-cold RIPA buffer (10 mM TRIS, 140 mM NaCl, 1% Triton-X100, 1% SDS, pH 7.4) in the presence of protease inhibitor (10 μl/g of cells), the soluble fraction was purified by two steps. The first step consisted of an His-Tag column (5 ml) using as solvent systems washing buffer (30 mM imidazole,

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50 mM HEPES, 300 mM NaCl, 5% glycerol, pH 7.5) and elution buffer (300 mM imidazole, 50 mM HEPES, 300 mM NaCl, 5% glycerol, pH 7.5). Protein fractions were pooled and dialyzed into 20 mM Tris pH 8.0 and 20 mM NaCl. The protein mixtures were loaded onto a 5-ml anion exchange column (HiTrap Q FF) and eluted with a linear gradient of 20 mM to 1 M NaCl using an AKTA FPLC system, to facilitate separation of glucosylated HMW1ct and HMW1C. Inclusion bodies were dissolved in guanidine hydrochloride (6 M) and separated from insoluble cell debris by centrifugation. Inclusion bodies purification was performed using His-Tag column (5 ml) with the same buffer system as washing buffer (guanidine·HCl, 6 M) and elution buffer (300 mM imidazole, 50 mM HEPES, 300 mM NaCl, 5% glycerol, pH 7.5). The obtained fractions were pooled, dialyzed to 0.1% PBS, then lyophilized, and stored in the freezer. The sequence of HMW1ct (Glc) is reported in Figure S7.

2.3 | MW-assisted solid-phase peptide synthesis

Peptides were synthesized by microwave-assisted solid-phase synthesis (MW-SPPS) following the Fmoc/*t*Bu strategy, using the Liberty Blue™ automated microwave peptide synthesizer (CEM Corporation, Matthews, NC, USA) following the protocol previously described.[19,20] Wang Tentagel® resin preloaded with the first amino acid was used (loading 0.23 mmol/g). Fmoc deprotections were performed with a solution of 20% piperidine in DMF (2 M). Peptide assembly was performed by repeating the MW-SPPS standard coupling cycle for each amino acid, using Fmoc-protected amino acids (2.5 equiv, 0.4 M in DMF), oxyma pure (2.5 equiv, 1 M in DMF), and DIC (2.5 equiv, 3 M in DMF). Uncertain peptide coupling steps were checked by the ninhydrin Kaiser[21] or microcleavages performed with a microwave apparatus CEM Discover™ single-mode MW reactor (CEM Corporation, Matthews, NC, USA). Final cleavage and side-chain deprotections were performed using a mixture of TFA/TIS/H_2O (95:2.5:2.5, v:v:v) at room temperature. After 2.5 h, the resin was filtered off, and the solution was concentrated flushing with N_2. The peptides were precipitated with cold Et_2O, centrifuged, and lyophilized. Deprotection of the hydroxyl functions of sugar moieties was performed by the addition of 0.1 M methanolic NaOMe solution to a solution of the lyophilized peptides in dry MeOH (1 ml/100 mg of resin) until pH 12 was reached. After 3 h, the reaction was quenched by the addition of concentrated HCl until pH 7; the solvent was evaporated under vacuum, and the residue was lyophilized. The crude peptides were purified by reverse-phase flash liquid chromatography on an Isolera One flash chromatography system (Biotage, Uppsala, Sweden) using a SNAP Ultra C18 column (25 g) at 20 ml/min as solvent systems H_2O (MilliQ) and ACN (gradients are reported in Table S1). The second purification of peptides was performed by semipreparative RP-HPLC on a Waters instrument (Separation Module 2695, detector diode array 2996) using a Sepax Bio-C18 column (Sepax Technologies, Newark, USA) (5 μm, 250 × 10 mm), at 4 ml/min with solvent systems A (0.1% TFA in H_2O) and B (0.1% TFA in ACN). Analytical characterization of the peptides was performed by analytical HPLC using a Waters ACQUITY HPLC coupled to a single quadrupole ESI-MS (Waters® ZQ Detector, Waters Milford, MA, USA) supplied with a BEH C18 (1.7 μm, 2.1 × 50 mm) column at 35°C, at 0.6 ml/min using solvent systems A (0.1% TFA in H_2O) and B (0.1% TFA in ACN). Gradient elution was performed with a flow of 0.6 ml/min and started at 10% B, with a linear increase to 90% B in 5 min. The analytical data, the chromatograms, and mass spectrometry spectra are reported in Figures S1–S6.

2.4 | Circular dichroism (CD)

CD spectra of peptides **4**–**6** and CSF114(Glc) were recorded using quartz cells of 0.1 cm path length with a JASCO J-710 CD spectropolarimeter at 25°C. The spectrum was measured in the 260–190 nm spectral range, 1 nm bandwidth, eight accumulations, and 100 nm/min scanning speed. The peptides were dissolved in PBS at a concentration of 100 μM. The secondary structure content of the peptides was predicted using the online server for protein secondary structure analyses BestSel.[22]

2.5 | Inhibition ELISA

Nunc-Immuno MicroWell 96-well polystyrene ELISA plates (NUNC Maxisorb, product code M9410, Merck, Milano, Italy) were coated with a solution 10 μg/ml, in pure carbonate buffer 0.05 M (pH 9.6) for peptide antigens or PBS (pH = 7.2) for protein HMW1ct(Glc), adding 100 μl/well, and incubated at 4°C overnight. Wells were washed (5×) using an automatic Hydroflex microplate washer (Tecan Italia, Milano, Italy) using washing buffer (0.9% NaCl, 0.05% Tween 20). Nonspecific binding sites were blocked with 100 μl/well of fetal bovine serum (FBS) buffer solution (10% in washing buffer) at room temperature for 1 h. Antibody apparent affinity was measured following the protocol for competitive ELISA previously reported.[23] Briefly, sera dilution was previously defined in titration curves at the semisaturating (i.e., 50%) absorbance value of 0.7. Seven different concentrations of each synthetic peptide or protein antigenic probe were used as inhibitors. Then, sera samples at the selected dilution were incubated in parallel with increasing concentrations of the antigenic probes (range 1×10^{-11} to 1×10^{-5} M) for 1 h at room temperature. All competitive experiments were performed in triplicate. After washes (3×), uninhibited antibodies were identified by adding 100 μl/well of alkaline phosphatase-conjugated with antihuman immunoglobulin G specific antibodies (IgG, Merck, Milano, Italy) diluted 1:3000 in FBS buffer. The microplates were then incubated 3 h at room temperature, and after washes (3×), 100 μl of substrate solution consisting of 1 mg/ml *p*-nitrophenyl phosphate (*p*NPP, Merck, Milano, Italy) and $MgCl_2$ 0.01 M in carbonate buffer (pH 9.6) were added. After approximately 30 min, the reaction was stopped with 1 M NaOH solution (50 μl/well), and the absorbance was read in a multichannel ELISA reader (Tecan Sunrise, Maennedorf, Switzerland) at 405 nm. Antibody titer values were calculated as (mean Abs of serum triplicate) − (mean Abs of blank triplicate) representing graphically the absorbance inhibition percentage. One positive and one negative serum, as references, were included in each plate for further normalization. Each experiment

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was performed at least twice in different days. Within-assays and between-assays coefficients of variations were below 10%. Calculated half maximal inhibitory concentration (IC_{50}) is reported for each antigenic probe. Coating conditions were set up independently for each peptide, and results are reported in Figures S8–S11.

The reference serum (MS14) was drawn from a relapsing–remitting MS patient, who had given informed consent, and was previously diagnosed after lumbar puncture, cerebrospinal fluid analysis, and MRI examination, fulfilling the established international diagnostic criteria.[24,25] This patient did not follow any specific treatment for the disease and showed high IgG antibody titers for years.

3 | RESULTS AND DISCUSSION

3.1 | Peptide synthesis

We synthesized the sequences previously selected by bioinformatic approach,[8] namely, FAN(635–655) (**1**), RTN4R(173–191) (**2**), and OMGp(162–180) (**3**) (Table 1), featuring sequence or structural homology with the peptide CSF114(Glc), designed synthetic antigenic probe for MS antibody identification, and characterized by the presence of a glucosylated Asn residue on the tip of a β-turn. We also prepared an *N*-glucosylated version of each peptide, that is, [Asn^{641}(Glc)]FAN(635–655) (**4**), [Asn^{153}(Glc)]RTN4R(147–165) (**5**), and [Asn^{168}(Glc)]OMGp(186–204) (**6**), because we know that the presence of the N-Glc moiety is fundamental for antibody recognition in the case of CSF114(Glc).

The glucopeptides **1**–**6** were synthesized by MW-SPPS, following the 9-fluorenylmethoxycarbonyl (Fmoc)/*t*Bu strategy and introducing the Fmoc-Asn[Glc (OAc)$_4$]-OH building block during the stepwise synthesis, as previously reported.[26] All the peptides were purified to homogeneity by reverse-phase flash liquid chromatography and analytically characterized by analytical HPLC coupled to a single quadrupole ESI-MS. Full analytical data are reported as Figures S1–S6.

TABLE 1 Sequences of FAN, RTN4R, OMGp and CSF114(Glc) peptides

Antigen	Fragment	Sequence
1	FAN(635–655)	GITVSR**N**GSSVFTTSQDSTLK
2	RTN4R(147–165)	TFRDLG**N**LTHLFLHGNRIS
3	OMGp(162–180)	TLINLT**N**LTHLYLHNNKFT
4	[Asn^{641}(Glc)]FAN (635–655)	GITVSRN(Glc)GSSVFTTSQDSTLK
5	[Asn^{153}(Glc)] RTN4R (147–165)	TFRDLGN(Glc)LTHLFLHGNRIS
6	[Asn^{168}(Glc)] OMGp (162–180)	TLINLTN(Glc)LTHLYLHNNKFT
	CSF114(Glc)	TPRVERN(Glc) GHSVFLAPYGWMVK

3.2 | CD

The conformation of the three glucosylated peptides **4**–**6** in water was explored by CD using PBS solutions (Figure 1). For comparison, peptide CSF114(Glc) was also considered in this analysis. The presence of a minimum around 200 nm, diagnostic of random coil conformation, indicated that peptides are highly flexible in this buffer. However, this minimum is more intense in the case of peptide **4** suggesting that this is the less ordered among the investigated peptides. In the case of peptides **5**, **6**, and CSF114(Glc), it can be observed a shift of the minimum from 199 to 200 (for peptide **6** and CSF114 (Glc)) and 201 nm (for peptide **5**) that, together with the already mentioned reduction of intensity, should point to some secondary structure stabilization. The secondary structure content (Table 2) was predicted based on the CD spectrum using the online server for protein secondary structure analyses BestSel.[22] As expected, all peptides have high percentage of unstructured conformation. The prediction also returned β-strand and β-turn structures that are the key elements of the β-hairpin in CSF114(Glc).[27,28] Notably, the percentage of β-strand content in the peptides is in the order **5** > **6** > **4** (Table 2).

3.3 | Inhibition ELISA

Inhibition ELISA experiments were performed to evaluate antibody cross-reactivity between the selected *N*-glucosylated peptides of myelin proteins and HMW1ct(Glc) (I(Glc)). The *N*-glucosylated peptides [Asn^{641}(Glc)]FAN(635–655) (**4**), [Asn^{153}(Glc)]RTN4R(147–165)

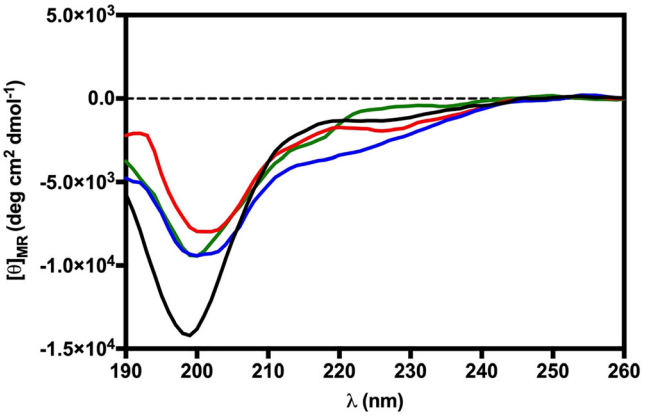

FIGURE 1 CD spectra of peptide **4** (black line), **5** (red line), **6** (blue line), and CSF114(Glc) (green line) in PBS solution

TABLE 2 Percentage of secondary structure from CD spectra

Peptide	α-Helix	β-Strand	β-Turn	Random coil
4	0.0	21.2	16.4	62.4
5	1.9	33.3	19.3	45.5
6	4.5	31.0	17.9	46.6
CSF114(Glc)	2.4	32.2	16.2	49.2

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(**5**), and [Asn^{168}(Glc)]OMGp(162–180) (**6**) were coated separately and the unglucosylated peptides **1**, **2**, and **3**; the glucopeptides **4**, **5**, and **6**; and the hyperglucosylated HMW1ct(Glc) protein I(Glc) were used as inhibitors at different concentrations with a reference MS serum diluted 1:100 (MS14), known to contain antibodies that recognize HMW1ct(Glc).

The results obtained (Figure 2) show that antibodies that recognize the *N*-glucosylated peptides **4**, **5**, and **6**, used as antigens on the plate, are inhibited by the hyperglucosylated protein HMW1ct(Glc) (I(Glc)). Moreover, self-cross-reactivity was observed for each one of the glucosylated peptides. On the other hand, unglucosylated peptides **1**, **2**, and **3** are not able to inhibit the antibody binding, thus confirming the fundamental role of the Asn(Glc) moiety as the minimal epitope recognized by MS sera.[6] The IC_{50} values reported in Table 3 show that the glucosylated peptide [Asn^{641}(Glc)]FAN(635–655) (**4**) is generally the weakest inhibitor (IC_{50}: $5.0{\cdot}10^{-7}$ M), while the glucosylated [Asn^{153}(Glc)]RTN4R(147–165) (**5**) is the strongest (IC_{50}: $3.0{\cdot}10^{-8}$ M). These results are in line with our previous observation that MS antibody binding to the designed glucosylated antigenic probe CSF114(Glc) can be inhibited by the hyperglucosylated NTHi bacterial adhesin HMW1ct(Glc), due to the presence of Asn(Glc), as part of a shared epitope.[17]

We subsequently performed the inhibition experiments coating the plates with the hyperglucosylated adhesin HMW1ct(Glc) (I(Glc)) and using all the peptides as inhibitors, in order to verify if the cross-reactivity between the glucosylated peptides and the hyperglucosylated protein was observed also when the latter high affinity antigenic probe is coated on the plate, while the peptide inhibitors are in solution. We preliminarily observed that the three unglucosylated peptide sequences, lacking Asn(Glc), were not able to inhibit MS serum antibodies recognizing HMW1ct(Glc).

Subsequently, as shown in Figure 3, we demonstrated that only the two glucosylated peptides [Asn^{153}(Glc)]RTN4R(147–165) (**5**) and [Asn^{168}(Glc)]OMGp(162–180) (**6**) were able to cross-react with Abs that recognize HMW1ct(Glc), inhibiting the antibody binding with an IC_{50} of $9.96{\cdot}10^{-7}$ and $2.73{\cdot}10^{-7}$ M, respectively (Table 3), thus confirming the presence of a shared epitope. At variance, the glucopeptide [Asn^{641}(Glc)]FAN(635–655) (**4**) was not able to inhibit antibody

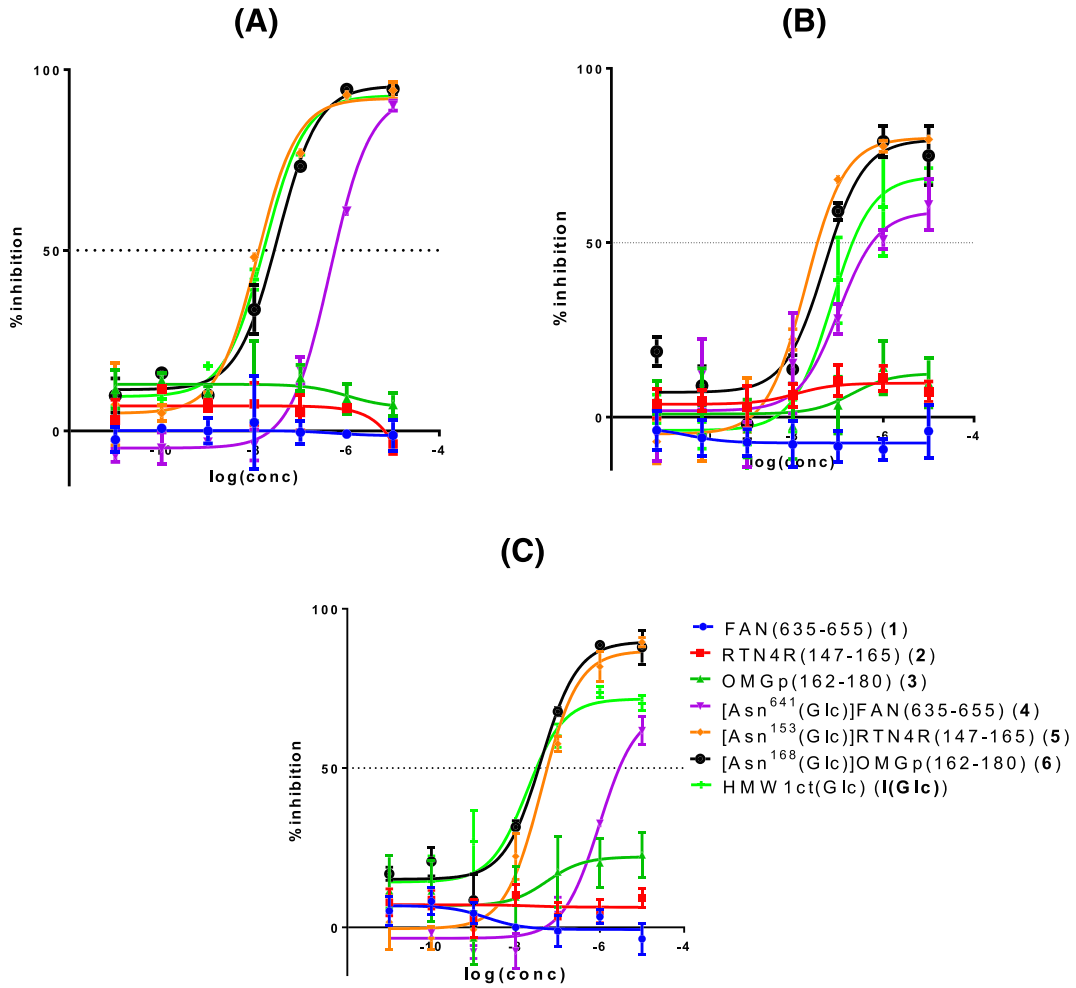

FIGURE 2 Inhibition of binding of MS14 serum IgG antibodies to (A) [Asn^{641}(Glc)]FAN(635–655), (B) [Asn^{153}(Glc)]RTN4R(147–165), and (C) [Asn^{168}(Glc)]OMGp(162–180) by peptides **1**, **2**, and **3**; glucopeptides **4**, **5**, and **6**; and hyperglucosylated HMW1ct(Glc) protein I(Glc) at different concentrations

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binding. Interestingly, this cross-reactivity was displayed only by the two glucopeptides **5** and **6** selected by a bioinformatic approach based on structural homology and not by the third one, that is, [Asn^{641}(Glc)]FAN(635–655) (**4**), selected by a mere sequence homology with CSF114(Glc). Notably, CD measurements indicate that peptides **5** and **6**, as well as peptide CSF114(Glc), feature an higher percentage of β-strand content, while peptide **4** is mainly in a random coil conformation, thus confirming the possible conformational homology between these two peptides (**5** and **6**) and CSF114(Glc) and characterized by a β-hairpin structure.[6] This important observation indicates that the MS-specific epitope present in the hyperglucosylated bacterial NTHi adhesin is significantly recapitulated by the synthetic probe CSF114(Glc) and is also present in the two human myelin proteins RTN4R and OMGp. Moreover, it suggests that the resemblance among the MS-specific epitopes is not limited to the Asn(Glc) moiety but has a significant conformational component. The latter aspect is evident only when the antigen is tested as an inhibitor in solution, where it is free to adopt the preferred conformation and expose it to Ab binding, and not when it is coated on the plate. In fact, the surface of the peptide which is involved in the adsorption interaction with the solid support is unavailable for binding to the antibody, and moreover, the adsorption of the peptide antigen to the plate may perturb its conformation. For these reasons, the antigenicity of a plate-bound peptide tends to be lower than that of the same peptide free in solution.[29] Accordingly, up to now, we have never been able to inhibit antibody binding to the hyperglucosylated adhesin using simple *N*-glucosylated peptides, unless they were assembled in structures bearing in the same molecule several copies of the antigen, to take advantage of multivalency.[19,30]

TABLE 3 Calculated IC_{50} values of MS14 serum IgG antibodies to [Asn^{641}(Glc)]FAN(635–655), [Asn^{153}(Glc)]RTN4R(147–165), [Asn^{168}(Glc)]OMGp(162–180) or HMW1ct(Glc) using as inhibitors peptides **4**–**6** and protein I(Glc)

Antigen coated	Inhibitor	IC_{50} (IgG)
[Asn^{641}(Glc)]FAN (635–655) (**4**)	[Asn^{641}(Glc)]FAN (635–655) (**4**)	$(4.35 \pm 0.06)\cdot10^{-7}$
	[Asn^{153}(Glc)]RTN4R (147–165) (**5**)	$(1.17 \pm 0.09)\cdot10^{-8}$
	[Asn^{168}(Glc)]OMGp (162–180) (**6**)	$(3.21 \pm 0.09)\cdot10^{-8}$
	HMW1ct(Glc) (I(Glc))	$(1.68 \pm 0.08)\cdot10^{-8}$
[Asn^{153}(Glc)]RTN4R (147–165) (**5**)	[Asn^{641}(Glc)]FAN (635–655) (**4**)	$(1.04 \pm 0.33)\cdot10^{-7}$
	[Asn^{153}(Glc)]RTN4R (147–165) (**5**)	$(1.93 \pm 0.07)\cdot10^{-8}$
	[Asn^{168}(Glc)]OMGp (162–180) (**6**)	$(5.03 \pm 0.26)\cdot10^{-8}$
	HMW1ct(Glc) (I(Glc))	$(7.38 \pm 0.13)\cdot10^{-8}$
[Asn^{168}(Glc)]OMGp (162–180) (**6**)	[Asn^{641}(Glc)]FAN (635–655) (**4**)	$(9.81 \pm 0.16)\cdot10^{-7}$
	[Asn^{153}(Glc)]RTN4R (147–165) (**5**)	$(4.10 \pm 0.11)\cdot10^{-8}$
	[Asn^{168}(Glc)]OMGp (162–180) (**6**)	$(4.01 \pm 0.15)\cdot10^{-8}$
	HMW1ct(Glc) (I(Glc))	$(1.89 \pm 0.24)\cdot10^{-8}$
HMW1(Glc) (I(Glc))	[Asn^{153}(Glc)]RTN4R (147–165) (**5**)	$(2.73 \pm 0.17)\cdot10^{-7}$
	[Asn^{168}(Glc)]OMGp (162–180) (**6**)	$(9.96 \pm 0.23)\cdot10^{-7}$
	HMW1ct(Glc) (I(Glc))	$(6.82 \pm 0.21)\cdot10^{-9}$

Note: Values are reported as 95% confidence interval for the calculated mean IC_{50} ± the standard error (SEM).

Most importantly, the demonstration of the presence of an epitope specifically recognized by MS patient sera and shared among CSF114(Glc), the hyperglucosylated bacterial adhesin, and the glucosylated peptides derived from two relevant human myelin proteins, that is, RTN4R and OMGp, strongly suggests that the latter proteins may be considered as MS autoantigens. Further experimental work is in progress to confirm this hypothesis and in particular to clarify the potential origins and the role of glucosylation of these antigens, because it is well known that this cotranslational modification should be considered aberrant, when found in human proteins.

In conclusion, we have shown that MS patient antibodies recognizing two glucopeptides derived from human myelin proteins that were selected on the basis of their conformational homology with the

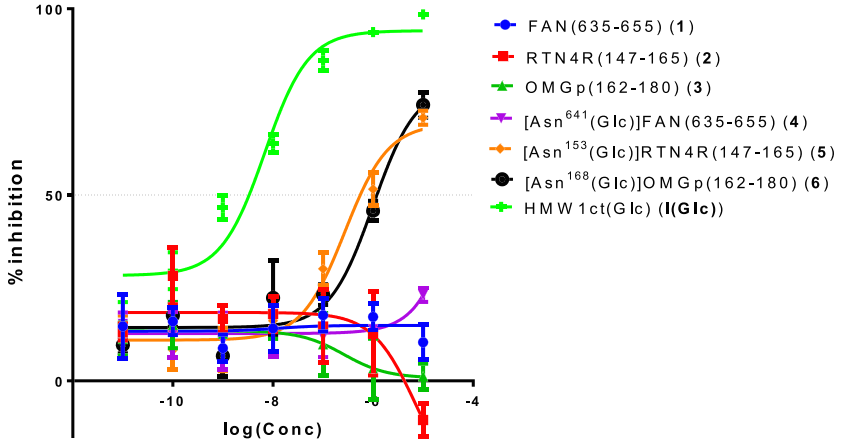

FIGURE 3 Inhibition of binding of MS14 serum IgG antibodies to HMW1ct(Glc) by peptides **1**, **2**, and **3**; glucopeptides **4**, **5**, and **6**; and hyperglucosylated HMW1ct(Glc) protein I(Glc) at different concentrations

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synthetic designed antigenic probe CSF114(Glc), cross-react with the recombinant hyperglucosylated nontypeable *H. influenzae* adhesin HMW1ct(Glc). This observation adds relevant evidence in support of two essential concepts highly debated in the recent literature concerning MS, but not fully explored: the involvement of sugar moiety, that is, *N*-glucosylated asparagine residues, and the contribution of infective agents (bacteria and/or viruses) to the disease etiology. In fact, our results strongly support the immunological resemblance between human and bacterial glucosylated protein antigens, underpinning the hypothesis that molecular mimicry triggers the breakdown of self-tolerance in MS.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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