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Naturally presented peptides on MHC I and II molecules eluted from central nervous system of multiple sclerosis patients

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Abbreviations

APC, antigen presenting cell

CNS, central nervous system

ESI-MS, electrospray ionization mass spectrometry

MBP, myelin basic protein

MOG, myelin oligodendrocyte glycoprotein

MS, multiple sclerosis

PLP, proteolipid protein

Q-TOF, hybrid quadrupole orthogonal acceleration time-of-flight

SSO, sequence specific oligonucleotides typing

TCR, T cell receptor

Summary

Tandem mass spectrometry was used to identify naturally processed peptides bound to MHC I and MHC II molecules in central nervous system (CNS) of eight patients with multiple sclerosis (MS). MHC molecules were purified from autopsy CNS material by immunoaffinity chromatography with mAb directed against HLA-A, -B, -C and HLA-DR. Subsequently, peptides were separated by reversed-phase HPLC and analyzed by mass spectrometry. Database searches revealed 118 amino acid sequences from self proteins eluted from MHC I molecules and 191 from MHC II molecules, corresponding to 174 identified source proteins. These sequences define previously known and potentially novel autoantigens in MS possibly involved in disease induction and antigen spreading. Taken together, we have initiated the characterization of the CNS expressed MHC ligandome in CNS diseases and were able to demonstrate the presentation of naturally processed MBP peptides in the brain of MS patients.

Introduction

T cells recognize antigen bound to MHC molecules (1). CD4 as well as CD8 T cells have been shown to play a pathogenic role in various autoimmune diseases (2). Pathogenic T cells infiltrate the target organs and locally secrete proinflammatory cytokines and chemokines leading to tissue inflammation and possibly subsequent tissue destruction (3-5). Local presentation of autoantigens by MHC molecules in the target tissue of the autoimmune attack i.e. the central nervous system (CNS) in multiple sclerosis (MS) or the pancreas in diabetes is therefore a prerequisite for local immune amplification (6). MS is an inflammatory and neurodegenerative disease of the CNS leading to myelin and axonal loss (7). There are different disease courses, i.e. relapsing remitting, secondary chronic progressive and primary progressive disease. Potential autoantigens in MS include myelin basic protein (MBP), proteolipid protein (PLP) and myelin oligodendrocyte glycoprotein (MOG). It is thought that T cells enter the CNS from the systemic circulation and that they are subsequently reactivated in the CNS on MHC I and MHC II molecules expressed on local antigen presenting cells (APC) (8).

To date, naturally presented HLA-bound peptides from patients with MS thus far have not been isolated and identified. So far, only circumstantial evidence exists for the local presentation of autoantigens such as MBP on MHC molecules in CNS (9). The aim of this study consisted of the characterization of the MHC-bound peptide repertoire derived from brains of patients with MS. Cutting edge technology combining HPLC and tandem mass spectrometry has recently allowed us to define peptides presented on APC from bronchoalveolar lavage from lungs of sarcoidosis patients (10). Applying a similar method on autopsy material of MS patients, for the first time, we demonstrate local presentation of previously known and potential novel autoantigens in MS.

Experimental procedures

Patient characterization

Brain autopsy samples from 8 patients with different clinical subtypes of MS were provided by the UK Multiple Sclerosis Tissue Bank (supplementary Table 1).

Elution of HLA-presented peptides

HLA-associated peptides were obtained by immunoprecipitation of HLA molecules from brain tissue of MS patients according to standard protocols (11, 12), using the HLA-A–, HLA-B–, and HLA-C–specific antibody W6/32 (13); and the HLA-DR–specific antibody L243 (14). In brief, about 50 g of snap frozen brain tissue was thawed at 4°C in PBS/ containing 1.2% (w/v) 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) in the presence of protease inhibitors (Complete Protease Inhibitor Cocktail Tablet, Roche Molecular Biochemicals) and homogenized in a Dounce homogenizer. Subsequently, HLA molecules and bound peptides were isolated with the solid-phase bound monoclonal antibody by immunoaffinity chromatography. Precipitates were eluted with 0.2% trifluoroacetic acid, ultrafiltrated by a 10-kDa ultrafilter and lyophilized.

Liquid chromatography-mass spectrometry

Lyophilized samples were resuspended in 150 µl solvent A (0.1% (vol/vol) trifluoroacetic acid water) and loaded onto a C18 precolumn (Dionex) for concentration and desalting with a flow rate of 50 µl/min. The precolumn was placed in line for separation by a 75µm ID fused-silicia microcapillary column packed with C18 RP material (Dionex). A binary gradient of 10%–55% solvent B (0.1% formic

acid in 80% [vol/vol] acetonitrile water) within 90 minutes was performed, applying a flow rate of 200-300 nl/min.

The peptides were analysed by electrospray ionization mass spectrometry (ESI-MS) on a hybrid quadrupole orthogonal acceleration time-of-flight (Q-TOF) tandem mass spectrometer. A gold-coated glass capillary (PicoTip) was used for introduction into the ESI source. A potential of 2.2 kV was applied to the capillaries, resulting in sample flow rates of 20-30 nl/min. The cone voltage was 60 V. A quadrupole analyzer was used to select precursor ions for fragmentation in the collision cell. The collision gas was argon used at collision energies of 24-50 eV. MHC I bound peptides were detected with a mass/charge (m/z) range of 400-800 and double charge, whereas MHC II bound peptides were measured in a m/z range of 500-1200 and double and triple charge.

Database searches

In tandem mass spectrometry experiments, sequence information was obtained by manual interpretation of fragment spectra using computer-assisted database (NCBInr, nonredundant protein database) searching tools (MASCOT, Matrix Science Mascot search version version 2.2; release February 28, 2007) (15). For peaklist generation MassLynx 4.0 with Service pack 2 was used. MASCOT searches were performed using *Homo sapiens* as taxonomy, no enzymatic specificity, ±0.3 Da for mass spectrometry tolerance, and ±0.3 Da for tandem mass spectrometry tolerance. These were done with database NCBInr 20070926 (5519594 sequences, 1911975371 residues), taxonomy homo sapiens (human) (194674 sequences); database NCBInr 20080110 (5828094 sequences; 2009144511 residues), taxonomy homo sapiens (human) (199003 residues); database NCBInr 20080610 (6573034 sequences; 2244863856 residues), taxonomy homo sapiens (human) (205031

sequences). The evaluations were performed over a longer time period, therefore all versions were used. Fragment spectra from synthetic peptides were recorded and compared with spectra from the corresponding eluted natural peptides to prove their identity.

Epitope prediction

For every peptide a prediction of the binding strength to the HLA type of the patients, was performed using the matrix-based SYFPEITHI algorithm. The prediction score is explained in detail in the information section of SYFPEITHI (http://www.syfpeithi.de).

HLA-genotyping

DNA for HLA genotyping was extracted from brain tissue using the QIAamp DNA Blood Mini Kit (Qiagen). Sequence specific oligonucleotides typing (SSO) was performed using the INNO-LiPA test (Innogenetics).

Results

Proteomic analysis of the MHC-I and MHC-II ligandome in the CNS

Brain autopsy samples from patients with different clinical subtypes of MS (supplementary Table 1) and different HLA haplotypes (supplementary Table 2) were analyzed using liquid chromatography tandem mass spectrometry. We isolated HLA-A, -B, -C and -DR molecules by affinity purification and then eluted the bound peptides by acid elution. Subsequently, peptides were separated by reversed phase HPLC and analyzed by mass spectrometry.

The tandem mass spectrometry-based peptide sequencing of HLA ligands extracted from brain yielded approximately 34 different ligands per patient. Table 1 shows the HLA class I- and class II-associated ligands isolated from MS patient MS1. Sequence analysis by tandem mass spectrometry allowed the identification of 11 MHC I and 23 MHC II bound peptides. We adopted the SYFPEITHI database to calculate the ligand binding affinity to the respective MHC I and II alleles according to the haplotype of patient MS1 (HLA-A*01, -A*03, -B*08 -B*51, -Cw*07 or -Cw*15 for MHC I and HLA-DRB1*03 or DRB1*04 for MHC II) (Table 1). For eight out of eleven MHC I ligands an assignment to the presenting HLA I molecule was carried out according to relatively high scores between 22 and 37 for binding to HLA-A*01, -A*03 or -B*51 (SYFPEITHI score above 20 predicts sufficient affinity to bind to the respective HLA molecule. The SYFPEITHI scoring system evaluates every amino acid within a peptide. Optimal anchor residues are given the value 15, amino acids that are only slightly preferred in the respective position may be given the arbitrary value 1. Any value between 1 and 15 is possible, negative values are also possible for amino acids which are disadvantageous for the peptides binding capacity at a certain sequence position). HLA I molecules were not assigned to three ligands lacking the correct anchoring amino acids. By contrast, we assigned HLA-DRB1*03 or DRB1*04 to all MHC II

ligands according to the scores of SYFPEITHI, which were generally lower than those of MHC I ligands.

Using mass spectrometric analysis of eight investigated brains of MS patients, we altogether identified 309 peptides of which 118 were eluted from MHC I and 191 from MHC II molecules (supplementary Table 3). As already shown for patient MS1, higher binding scores were obtained for peptides restricted to MHC I molecules as compared to MHC II molecules.

Verification of the identified peptides

For confirmation of identified ligands, a subset of these peptides was synthesized in order to prove their identity. Mass spectrometric analysis was performed on the synthetic peptides and obtained fragment spectra were compared with the corresponding spectra of the naturally presented peptides eluted from brain tissue. Fig. 1 shows an example of the fragmentation spectra of two naturally occurring peptides, neurofilament light chain $NEFL_{72-84}$ (DLSQVAAISNDLK) (Fig. 1A) eluted from MHC I molecules in samples of patients MS3 and MS6 and glutamine synthetase (LNETGDEPFQYKN) (Fig. 1B) obtained from MHC II molecules in MS samples of patients MS1, MS4, MS5, MS7 and MS8. In all cases, spectra of the synthetic peptides matched the ones of their eluted natural counterparts (Fig. 1C and 1D), proving the correct interpretation of the recorded mass spectrometric data.

Database mining

The analysis of the constitutive HLA peptide repertoire presented by various HLA allotypes in brains of patients with MS allowed us to identify 239 non-redundant peptides derived from 174 non-redundant source proteins. Among these, we were able to identify several HLA ligands from 40 proteins that have previously been

reported to be associated with MS or its animal model, experimental autoimmune encephalomyelitis (EAE) (Table 2). These proteins could be categorized regarding their function and tissue localization into proteins related to apoptosis, cytoskeleton, enzymatic reactions, immune responses, CNS, serum and others (Table 2).

Ingenuity Pathways Analysis® of these 174 source proteins revealed that the largest quantity of eluted peptides were derived from proteins present in the cytoplasm (34%) followed by proteins derived from plasma membranes (25%), nucleus (17%), unknown origin (13%) and extracellular space (11%) (Fig. 2A). 68% of the eluted peptides derived from extracellular proteins were presented on MHC II molecules, 77% of the peptides derived from nuclear proteins were presented on MHC I molecules. In contrast plasma membrane and cytoplasm derived peptides were equally presented on MHC I and II molecules.

In addition, the program was used to link the proteins with different diseases. Particularly relevant for this study were proteins involved in neurological diseases (28%) and also proteins associated with inflammatory and immunological diseases (8%), moreover the analysis showed a high number of proteins involved in other diseases as well as a considerable number of proteins present in more than one group (Fig. 2B).

With regard to their biological functions, the source proteins of HLA ligands could be categorized into ten partially overlapping functional groups (Fig. 2C). 35 of the source proteins participate in cellular assembly and organization, 26 are involved in cellular movement, 25 in nervous system development and function, 19 are associated with cell death, 16 with cell cycle, 13 with molecular transport, ten with cellular growth and proliferation, ten with cell-to-cell signaling and interaction, six with gene expression and six with immune responses.

MHC bound MBP peptides

MBP is one of the most abundant myelin proteins. It is the best studied myelin protein in MS, which is partly due to the finding that MBP is immunogenic and that MBP-specific T lymphocytes have encephalitogenic activity in experimental animals (16). It is known that T cells recognize a large number of MBP epitopes, restricted by different HLA-molecules (17-19). This view is supported by our finding of MBP epitopes presented by different MHC molecules in 7 of the 8 analyzed patients (Table 3). We identified 2 HLA class I restricted and 11 HLA class II restricted amino acid sequences derived from MBP. MBP-derived HLA class I peptides were identified in 2 out of 8 MS patients, whereas MBP-derived MHC class II peptides could be identified in 5 out of 8 MS patients (Table 3).

Discussion

In this study, we identified naturally processed peptides presented on MHC I and II molecules eluted from brains of patients with MS. To our knowledge, it is the first time that this approach has been successfully applied to a CNS disease. MS is an inflammatory disease that leads to neurodegeneration (7). Early inflammatory events trigger subsequent events leading to demyelination, axonal and neuronal loss and finally scar formation. So far, the autoimmune response has been considered to be mainly directed against myelin antigens, like MBP (20). There are some indications that also axonal and neural components or astrocytic proteins could be auto antigens in MS (21). In this study we clearly demonstrate that self-antigens are presented in the course of MS that have been known for a long time, like MBP peptides (19, 22-26). We found peptides derived from glial fibrillary acidic protein (GFAP) and neurofilament that have been shown to be enhanced in cerebrospinal fluid (CSF) of MS patients (27). Furthermore, immunization with neurofilament can cause neurological disease and axonal damage in mice (28). The first example of immunological self-mimicry has been recently demonstrated for neurofilament (29). MOG-deficient mice that express a MOG35-55 specific transgenic T cell receptor (TCR), recognize an epitope of the medium-sized neurofilament NF-M and develop disease. The authors propose that the combined immune response to the two target structures by encephalitogenic T cells might be a reason to overcome resistance to EAE in C57BL/6 mice (29). Immunological self-mimicry could be of paramount importance in the pathogenesis of MS and induction of neurodegeneration.

In Table 2 we give an overview about the proteins which have been demonstrated to be of potential importance in MS. For example, these proteins are known to be biomarkers in MS, like actin (27), targets for autoantibodies, like glyceraldehyd-3-phosphate dehydrogenase (30) or have an effect in animal models of MS, e.g. early

pregnancy factor (31), or might be of diagnostic value, like gelsolin (32). Further analysis is necessary to clarify if these proteins, and especially the identified peptides, are disease relevant in MS. In addition we define many novel potential autoantigens derived from several cell types that have not been described in the context of MS. The recent description of double self-reactive T cells in EAE implicates that our identified peptides could be as well targets of such double-reactive T cells in MS (29). Therefore they should be closely examined regarding shared TCR contact positions with known immunodominant epitopes, e.g. MBP85-99.

Various studies suggest that MBP is a candidate autoantigen in MS. The MBP 85-99/MHC complex has been demonstrated to be present in the CNS of MS patients with a monoclonal antibody (9). Evidence for the functional importance comes from the observation that transgenic mice expressing both the HLA-DR1*1501 molecule and an MBP 84-102 specific TCR, derived from an MS patient, spontaneously develop EAE (33). Extensive epitope mapping studies have been conducted in animals as well as in humans, to identify potentially encephalitogenic MBP sequences. Many studies have identified MBP 83-99 as immunodominant in EAE as well as in MS (34). This peptide promiscuously binds to various MS-associated HLA-DR molecules. However, it has been suggested that the unusually high binding affinity of this peptide to HLA-DR2 molecules might lead to the deletion of high-avidity MBP 83-99 specific T cells from the peripheral T cell repertoire. Although the role of MBP 84-102 in MS has been widely investigated, other MBP-derived epitopes have equally been shown to be immunodominant and might be more discriminatory between patients and healthy controls (35, 36). These peptides include MBP 14-33 and the HLA-DR1*0401 associated peptide MBP 113-131.

HLA class I restricted MBP-peptides are less well characterized as the strongest genetic association of MS is with HLA class II alleles and only to a lesser extent with HLA class I alleles (37). Therefore, MS is generally considered to be a CD4+ T cell driven disease. Nevertheless, CD8+ T cells have recently moved to the focus of attention in MS research, as it was shown that CD8+ T cells are prominently present in MS lesions and are more clonally expanded in these lesions as CD4+ T cells (38). There is some indication that HLA-A2 may be protective and HLA-A3 disease promoting in MS (39-41). Immunodominant HLA-A2 restricted MBP peptides include MBP 88-96 and MBP 112-120 (42). The two different HLA class I restricted MBP sequences are overlapping (34-44 and 35-44) and consist of MBP sequences which have not previously been associated with pathogenic CD8+ T cell responses in MS.

As far as the HLA-DR restricted MBP epitopes are concerned, most MBP naturally processed peptides originate from regions that have been previously been implicated in MBP-immunodominance (34). Thus, four MBP peptides (MBP 10-19, MBP 10-27, MBP 11-25 and MBP 21-30) are partially overlapping with the MBP 14-33 immunodominant epitope. Several naturally processed MBP peptides partially overlap with the MBP 84-102 region (Table 3). Interestingly, none of the peptides identified in this region completely corresponded to the very well characterized MBP 83-99 epitope. As a matter of fact, for two of the patients, the MBP epitopes represented contiguous sequences (MS1: MBP 84-94 and MBP 95-106; MS2: MBP 84-94 and MBP 95-110/ 95-112) within this region, which were cleaved between Asn94 and Ile95. Interestingly, it has been previously shown that this site in MBP represents a cleavage site for asparagine endopeptidase (AEP), an enzyme important for early stage proteolysis during antigen processing (43) (Table 4). Thus, our results strongly support a biological role for AEP in MBP processing in the brain of MS patients. Studies in SJL/J mice have shown that induction of immunological

tolerance with MBP 89-101 is ineffective, as in these mice, this treatment does not target the disease-relevant T cells which recognize either MBP 89-94 or MBP 95-101 (44). These observations made in an EAE model in conjunction with our data strengthen the notion that relevant antigen-specific MS therapies should be designed in such a way that they target T cells responsive to naturally processed epitopes of the autoantigen.

We did not detect peptides derived from other well known myelin proteins, such as peptides from PLP and MOG. This is possibly due to the density of the presented peptides on MHC molecules. PLP is the most abundant myelin protein (50%), followed by MBP (40%). In contrast, MOG only makes up 0.01 to 0.05% of myelin. The reason why no PLP peptides nor MOG peptides where identified might be twofold. As far as MOG peptides are concerned, the low abundance of MOG in the CNS might hamper the detection of MOG-derived peptides. In contrast, lack of detection of PLP-derived peptides might be due to the fact that PLP is generally highly palmitoylated, as current methodologies are still limited in their identification of such modified peptides.

As shown, we analyzed only CNS tissue from patients with MS and not from healthy controls. There are several reasons for this. Firstly, the availability of CNS tissue especially from healthy and young individuals is very limited. Secondly, the comparison between healthy and control tissue would only allow conclusions in MHC haplotype matched controls. Interestingly, this point is underscored by the finding that common peptides could be eluted from APC in lungs of patients with sarcoidosis and brain tissue from MS patients that expressed HLA-DRB1*0301 (10) (Table 5). Thirdly, MHC II is mainly expressed on professional APC and expression is strong under pathological conditions in MS (45). In contrast in healthy CNS, MHC expression is low. This argument is strongly supported in mice immunized with either

MOG in CFA or PBS in CFA in which the number of isolated peptides out of the same amount of brain tissue is decreased more than 70 % in PBS immunized mice (unpublished data Sabrina Haag). The minimal number of cells allowing the analysis of HLA-bound peptides is approximately 10^9 cells. In order to reach this amount of MHC II expressing cells from CNS of healthy controls it would be necessary to increase the amount of tissue strongly.

Although many peptides presented by MHC I and II molecules have a significant binding affinity score, we observed HLA ligands with low scores. This is unexpected for peptides presented in MHC I molecules due to the distinct peptide motif of these proteins. A possible explanation is that the SYFPEITHI program does not cover all different HLA alleles, like e.g. HLA-C, HLA-DR B1*13.

Unlike traditional proteomic approaches (46), our approach has the clear advantage to be able to define stretches of proteins that are visible to the immune system and are therefore more likely to be involved in an immune response as peptides defined by classical epitope mapping studies.

In addition, the presented MHC ligandome mirrors the proteome that is involved in the disease process. We identified peptides that mirror all the different features of the MS disease process like neurodegeneration, apoptosis, remyelinatinon. GFAP, tubulin and actin are biomarkers for neurodegeneration; annexin, bcl-2 associated transcription factor 1 and survivin are apoptosis related proteins and microtubule-associated protein 1B is a marker for actively myelinating oligodendrocytes. Therefore, we believe that disease-relevant information can be generated based on these data, by looking at B- and T cell responses to peptides, and the proteins they are derived of, in the course of MS. Data generated accordingly, might allow to better define the disease-driving autoantigens in MS. In addition such data can be used for definition of novel biomarkers (47). The eluted peptides are highly interesting in

regard to antigen spreading that has been involved in chronicity of autoimmune diseases. It will be a major challenge to define the hierarchy and timing of spreading of the eluted peptides towards T cell epitopes in MS patients. It has been shown that induction of tolerance against autoantigens presented on MHC molecules has a great therapeutic potential in experimental autoimmune diseases (20, 48). Therefore the eluted sequences are also of great interest for the design of tolerogens capable of preventing or treating MS.

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Figure Legends

Fig. 1. Examples of fragmentation-induced mass spectra. Mass spectra of the naturally eluted peptides (**A**) neurofilament light polypeptide, DLSQVAAISNDLK and (**B**) glutamine synthetase, LNETGDEPFQYKN in comparison to the corresponding synthetic peptides (**C** and **D**) recorded with a Q-TOF mass spectrometer. The corresponding y and b series are marked.

Fig. 2. Subcellular, functional and disease-associated categorization of source proteins by Ingenuity Pathways Analysis®. (**A**) Pie chart shows the percentages of proteins according to their subcellular localization. (**B**) Venn diagram depict the association of the proteins to neurological, inflammatory, immunological and other diseases. (**C**) Pie chart illustrates the functional association of the proteins.

Table 1: MHC class I and II ligands identified from one individual MS patient (MS1). Identified peptides from patients MS2-MS8 are shown in supplementary tables.

MS1
HLA typing: HLA class I: A*01, A*03, B*08, B*51, Cw*07, Cw*15
HLA class II: DRB1*03, DRB1*04, DQB1*0302, DQB1*0201, (DQB1*0204)

No.	Entrez gene ID	Source protein	m/z	Charge	Mascot score	% secuence coverage	Peptide sequence	HLA assignment (SYFPEITHI
MHC class I restriction								
1	118	alpha-adducin	584.25	2	48	6	LTDRELEEY	A*01(33)
2	147463	ankyrin repeat domain-containing protein 29	506.78	2	53	3	VIRLLLASGA	NA
3	256949	ankyrin repeat domain-containing protein 47	598.88	2	43	4	ALMLAISHGRQ	NA
4	311	annexin A11	677.25	2	71	7	ETDLLDIRSEY	A*01(34)
5	3481	insulin-like growth factor II precursor	488.26	2	37	5	IPMGKSMLV	B*5101(22)
6	8543	LIM domain only protein 4	569.82	2	42	5	KIADRFLLY	A*03(24)
7	51604	phosphatidylinositol glycan	615.22	2	52	3	DTDHYFLRY	A*01(37)
8	4735	septin 2	631.77	2	58	5	YIDEQFERY	A*01(30)
9	9201	serine/threonine-protein kinase DCLK1	653.21	2	71	1	YTERDASGMLY	A*01(31)
10	85358	SH3 and multiple ankyrin repeat domains protein 3	515.77	2	41	<1	CPLSLAAQLD	NA
11	7401	USH3A (clarin 1)	564.26	2	46	33	TTGILSILFY	A*01(24)
MHC class II restriction								
1	60	actin	677.71	3	113	10	WISKQEYDESGPSIVHR	DRB1*0401(18)
2	3040	alpha-2-globin	506.30	4	77	13	AAHLPAEFTPAVHASLDKF	DRB1*0401(28)
3	347	apolipoprotein D	822.45	2	73	7	NQELRADGTVNQIEG	DRB1*0401(26)
4	3043	beta-globin	623.70	3	107	65	YQKVVAGVANALAHKYH	DRB1*0401(26)
5	22883	calsyntenin 1	767.48	2	60	1	DPPLIALDKDAPLR	DRB1*0301(31)
6	1363	carboxypeptidase E	649.99	3	78	3	EPGEPEFKYIGNMHGNE	DRB1*0401(22)
7	83692	CD99 antigen-like 2	635.89	2	66	6	AEPPPPPPEPARI	DRB1*0301(9)
8	8760	CDP-diacylglycerol synthase 2	613.41	2	44	2	PEVLNRALSNL	DRB1*0301(21)
9	3336	early-pregnancy factor, EPF (chaperonin 10)	699.41	2	61	44	FRDGDILGKYVD	DRB1*0301(19)
10	2752	glutamine synthetase	777.89	2	71	3	LNETGDEPFQYKN	DRB1*0301(20)
11	2597	glyceraldehyde-3-phosphate dehydrogenase	845.95	2	85	4	YDNEFGYSNRVVDL	DRB1*0401(22)
12	3040	hemoglobin alpha-2	586.37	2	79	35	VLSPADKTNVK	DRB1*0401(20)
13	3903	leukocyte-associated Ig-like receptor (LAIR)	626.34	2	62	4	FRIDSVSEGNAG	DRB1*0301(20)
14	3916	lysosomal-associated membrane glycoprotein-1	786.00	2	44	3	LNTILPDARDPAFK	DRB1*0301(29)
15	3107	MHC class 1 protein HLA-C heavy chain	642.67	3	54	22	VDDTQFVRFDSDAASPR	DRB1*0401(28)
16	4155	myelin basic protein (MBP_{84-94})	673.39	2	57	6	DENPVVHFFKN	DRB1*0401(18)
17	4155	myelin basic protein (MBP_{95-106})	625.38	2	32	7	IVTPRTPPPSQG	DRB1*0401(14)
18	9378	neurexin 1	857.98	2	43	9	ESNAIINDGKYHVVR	DRB1*0301(28)
19	9379	neurexin 2	562.00	3	57	2	EPNAIVSDGKYHVVR	DRB1*0301(29)
20	113791	phosphoinositide-3-kinase	764.41	2	52	5	GHLYREDQTSPAPG	DRB1*0401(28)
21	84898	plexin domain containing 2	725.45	2	64	2	LDFLKAVDTNRAS	DRB1*0401(26)
22	8404	SPARC-like protein 1	573.06	3	52	2	KVKKIYLDEKRLLA	DRB1*0301(37)
23	6386	syntenin	709.92	2	71	4	ITSIVKDSSAARNG	DRB1*0301(24)

* Note: the scores of individual peptides calculated using the SYFPEITHI epitope

Prediction program are shown in brackets. (NA)= not assigned

Table 2: MS associated proteins. According to literature, some of the source proteins have been previously shown to be associated with MS. These could be categorized regarding the function and tissue localization.

Entrez ID	Protein	Patients	Association with MS
Apoptosis			
311	annexin A1	MS1, MS2, MS4, MS7	Identified as potential new marker of active MS disease (49)
9774	bcl-2 associated transcription factor 1	MS4, MS6	Anti-apoptotic molecule increased in MS cortical neurones (50)
332	survivin	MS2	Heightened expression in activated T cells from MS patients (51)
Cytoskeleton			
60	actin	MS1, MS2, MS4, MS5, MS6, MS7, MS8	Biomarker of axonal and neuronal damage (47)
10376	alpha-tubulin	MS4, MS5, MS6, MS7	Biomarker of axonal and neuronal damage (47)
2934	gelsolin	MS5	Lower gelsolin concentration in CSF of MS patients, indicating the possible utility for diagnostic purposes (32)
4131	microtubule-associated protein 1B, MAP1B	MS3	MAP1B is a marker for actively myelinating oligodendrocytes in adult rat brain (52)
3925	stathmin 1	MS4	Increased stathmin expression in the brains of MS patients (53)
Enzymes			
801	calmodulin 1	MS5	Calmodulin binds MBP in Ca^{2+}-dependent manner (54)
805	calmodulin 2 (phosphorylase kinase)	MS4, MS6	Involved in migration of gamma/delta T cells to the site of lesion in MS (55)
2747	glutamate dehydrogenase, GDH	MS3, MS5, MS3, MS5	Glutamate excitotoxicity, evoked by altered glutamate homeostasis, was demonstrated in an animal model of MS; GS and GDH are present in oligodendrocytes in normal and non-MS white matter, but were absent from active and chronic silent MS lesions, suggesting lasting metabolic impediments (30)
2752	glutamine synthetase, GS	MS1, MS3, MS4, MS5, MS7, MS8	Glutamate excitotoxicity, evoked by altered glutamate homeostasis, was demonstrated in an animal model of MS; GS and GDH are present in oligodendrocytes in normal and non-MS white matter, but were absent from active and chronic silent MS lesions, suggesting lasting metabolic impediments (30)
2597	glyceraldehyde-3-phosphate dehydrogenase	MS1, MS3, MS4, MS6, MS7	Autoantibodies in CSF of patients with MS (31)
51564	histone deacetylase 7a	MS6	Histone deacetylase inhibitors are discussed as a dual therapeutic modality in MS (56)
4696	NADH dehydrogenase	MS4, MS5	There is a trend for impaired NADH dehydrogenase activity in association with oxidative damage to mitochondrial DNA in chronic active plaques in MS (57)
113791	phosphoinositide-3-kinase, Pi3K	MS1	Patients with RR-MS display an increase in peripheral Vdelta2 gamma/delta T cells, they transmigrate across endothelial cells by activation of the Pi3K pathway (58)

Entrez ID	Protein	Patients	Association with MS
Immune response			
336	early pregnancy factor, EPF	MS1, MS3, MS4, MS5, MS8	EPF has a protective effect on EAE in LEW rats (59)
3577	interleukin 8 receptor, CXCR1	MS8	Treatment with methylprednisolone and mitoxantrone modulates the expression of CXCR1 in blood mononuclear cells (60); CXCR1 is constitutively expressed on oligodendrocytes and upregulated around active and silent lesions in MS patients (61)
3594	interleukin 12 receptor	MS12	Suggested as biomarker that can differentiate between relapsing remitting and secondary progressive MS stages (47)
4282	macrophage migration inhibitory factor	MS4	A therapeutic target in inflammatory diseases (62)
3119	MHC class antigen II HLA-DQ-beta-1	MS2	HLA-DR and –DQ genes are the strongest genetic risk factors in MS (20, 45)
CNS			
6622	alpha-synuclein	MS4	Upregulated in neurons and glia (63)
2670	glial fibrillary acidic protein, GFAP	MS2, MS5, MS6, MS8	Elevated in CSF of MS patients (27), biomarker of gliosis (47)
4155	myelin basic protein, MBP	MS1, MS3, MS6, MS7, MS8	Anti-MBP antibodies suggested for prediction of development of MS, biomarker od demyelination (47)
4741	neurofilament-3, NF-M	MS7, MS8	Biomarker of axonal and neuronal damage (47); autoantibodies in serum and CSF (64); transgenic MOG_{35-55} reactive T cells respond to an NF-M epitope in MOG deficient mice (29)
4747	neurofilament light polypeptide, NF-L	MS3, MS6	Biomarker of axonal and neuronal damage (47); elevated in CSF of MS patients (27); immunization with NF-L induces neurological disease and axonal degeneration in mice (28); autoantibodies in serum and CSF (65)
6285	S100 calcium-binding protein	MS2	Raised levels in CSF in RR- MS patients (66)
8404	SPARC-like protein 1	MS1, MS2, MS3, MS5	Present in the CSF of MS patients (67)
3925	stathmin 1	MS4	Increased expression of stathmin in CNS of MS patients (53)
Serum proteins			
2	alpha-2-macroglobulin	MS2, MS8	Significantly increased fractions of transformed to total alpha-2-macroglobulin in plasma from patients with MS (68, 69)
347	apolipoprotein D, apoD	MS2	Intrathecal apoD production is increased in MS, CSF apoD traces correlate with disease duration, corticosteroid treatment results in elevated CSF apoD levels (70)
348	apolipoprotein E, apoE	MS4	apoE-derived peptides ameliorate clinical disability and inflammmatory infiltrates in the spinal cord in EAE (71)
2512	ferritin	MS2, MS8	Elevated ferritin levels in MS patients with chronic progressive active disease (72); elevated levels of ferritin are found in a certain percentage of MS patients (73)
7018	transferrin	MS2	There is evidence for iron dysregulation in the pathogenesis of MS, opposite to the ferritin levels transferrin levels in patients are in a normal range (72)
7450	von Willebrand factor	MS7	$CXCR3^{(-/-)}$ mice with EAE show more von Willebrand factor-immunoreactive vessels within inflamed spinal cords, as compared with $CXCR3^{(+/+)}$ mice (74)

Entrez ID	Protein	Patients	Association with MS
Others			
2784	guanine nucleotide-binding protein, GNB	MS4	Trend to a increased GNB polymorphism in primary chronic progressive MS patients (75)
4600	interferon-regulated resistance GTP-binding protein (MX1)	MS3	Biomarker to measure IFN-beta activity in mice following gene-based delivery (76)
10226	mannose 6 phosphate receptor (IGF-II)	MS4	No expression in plaques of MS patients, which were characterized by a dense network of astrocytes, suggesting that IGF-II receptors in human brain are not involved in astrogliosis (77)
7157	P53 tumor suppressor	MS2	Defects in the ATM-CHK2-p53 pathway in MS patients likely alter the regulation of the immune population of cells in MS and may contribute to the development or progression of the disease (78); increased p53 expression in oligodendrocytes in active MS lesion feature oligodendrocyte apoptosis and cell loss (79)
7082	tight junction protein ZO-1	MS4	Abnormal tight junctions are most frequent in active white matter lesions but persist in inactive lesions of MS patients (80)

Table 3: Multiple peptides identified from MBP sequence. Diverse MBP peptides eluted in 7 out of 8 MS patients were presented on MHC I and II molecules.

Protein (Position)	Peptide sequence	Patient
MHC class I		
$MBP_{(35-45)}$	DTGILDSIGRF	MS6, MS7
$MBP_{(36-45)}$	TGILDSIGRF	MS7
MHC class II		
$MBP_{(10-19)}$	RHGSKYLATA	MS3
$MBP_{(10-27)}$	RHGSKYLATASTMDHARH	MS3
$MBP_{(11-25)}$	HGSKYLATASTMDHA	MS8
$MBP_{(21-30)}$	TMDHARHGFL	MS3
$MBP_{(77-91)}$	SHGRTQDENPVVHF	MS8
$MBP_{(84-94)}$	DENPVVHFFKN	MS1, MS3
$MBP_{(95-106)}$	IVTPRTPPPSQG	MS1, MS4
$MBP_{(95-110)}$	IVTPRTPPPSQGKGRG	MS3
$MBP_{(95-112)}$	IVTPRTPPPSQGKGRGLS	MS3
$MBP_{(96-111)}$	VTPRTPPPSQGKGRGL	MS5
$MBP_{(139-153)}$	HKGFKGVDAQGTLS	MS3

Table 4. Eluted peptides and AEP cleavage site.

											AEP																
MBP 84-112	**D**	**E**	**N**	**P**	**V**	**V**	**H**	**F**	**F**	**K**	**N**	**I**	**V**	**T**	**P**	**R**	**T**	**P**	**P**	**P**	**S**	**Q**	**G**	**K**	**G**	**R**	**G**	**L**	**S**	
MBP 84-94	D	E	N	P	V	V	H	F	F	K	N																			**MS 1**
MBP 95-106												I	V	T	P	R	T	P	P	P	S	Q	G							
MBP 84-94	D	E	N	P	V	V	H	F	F	K	N																			**MS 3**
MBP 95-110												I	V	T	P	R	T	P	P	P	S	Q	G	K	G	R	G			
MBP 95-112												I	V	T	P	R	T	P	P	P	S	Q	G	K	G	R	G	L	S	
MBP 95-106												I	V	T	P	R	T	P	P	P	S	Q	G							**MS 5**

Table 5: Common peptides eluted from CNS and lung of HLA-DRB1*0301 positive MS and sarcoidosis patients, respectively. Common peptides were mainly observed from patients carrying the same HLA-DR allele, HLA-DR*0301. The peptides are not identical, but overlapping and share a minimum of 10 amino acids.

Protein	Peptide sequence	Patients	Allele	Sarcoidosis patients
alpha-2-macroglobulin	SSKFQVDNNNRL	MS2, MS8	DRB1*0301, DRB1*1501	SSKFQVDNNNRL (10)
alpha-2-macroglobulin	GNRIAQWQSFQLEG	MS2	DRB1*1501	GNRIAQWQSFQLEG (10)
ATP synthase, H^+ transporting, F0 complex	PKFEVIEKPQA	MS7	DRB1*0301	PTFKFEDPKFEVIEKPQA (10)
Beta-globin	GKVNVDEVGGEALGRL	MS3, MS5, MS6	DRB1*0301, DRB1*0101	KVNVDEVGGEALGRL (10)
Beta-globin	GKVNVDEVGGEALGRLL	MS5	DRB1*0101	KVNVDEVGGEALGRLL (10)
Beta-globin	EVGGEALGRL	MS6	DRB1*0301	
Beta-globin	EVGGEALGRLL	MS6	DRB1*0301	
Beta-globin	VGGEALGRLL	MS7	DRB1*0301	
Beta-globin	VHLTPEEKSAVTALWGK VNVDEVGGEALGRL	MS8	DRB1*1501	
syntenin	ITSIVKDSSAARNG	MS1, MS2	DRB1*0301	ITSIVKDSSAARNGl (10)
transferrin	YAVAVVKKDSG	MS2	DRB1*0301	DPQTFYYAVAVVKKDSG (10)

Figure 1

Neurofilament light polypeptide

Glutamine synthetase

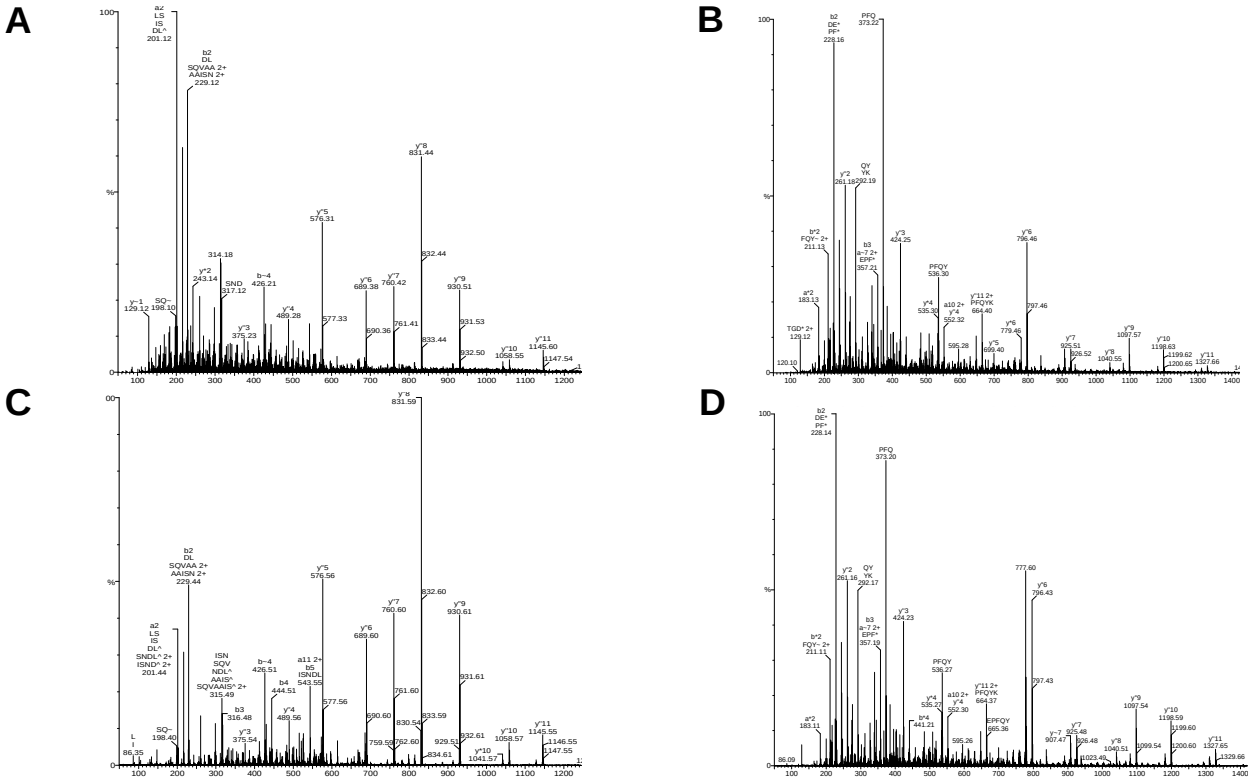

Figure 2