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To look for a needle in a haystack: the search for autoantibodies in multiple sclerosis

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Abstract

The search for autoantibodies in multiple sclerosis (MS) has been challenging for the last 3 decades. With the development of new proteomic methods and advances in expression and assay technologies, progress in the identification of MS autoantibodies has been made. A number of MS-specific autoantibodies have been proposed, most of them targeting proteins expressed in oligodendrocytes and along the myelin sheath. In this review, we summarize the status of antibody research in MS and then discuss recent developments and future strategies in defining and characterizing the potential antigenic targets of autoantibodies in MS.

Keywords

Autoantibodies, multiple sclerosis, pathology, proteomics, review

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Introduction

Autoimmune responses against proteins expressed in the central nervous system (CNS) are believed to play a crucial role in the pathogenesis of multiple sclerosis (MS).[1,2] Both arms of the adaptive immune response, antigen-specific T and B cells, seem to contribute to the development of MS lesions. The importance of the humoral immune response is suggested by the occurrence of antibody-mediated complement activation in some acute MS lesions and the beneficial effect of plasma exchange in MS patients with this pattern of pathology.[3,4] The beneficial effect of therapeutic B-cell depletion on disease activity also argues for a role for B cells.[5,6] While T and B cells in lesions and cerebrospinal fluid (CSF) show signs of an antigen-driven immune response towards CNS antigens, the targets of the immune responses have largely remained unclear.[7] During the last decades, many studies have addressed the specificity of T cells and antibodies. Here we review the technical challenges and the current status of antibody research in MS.

Methods to determine autoantibody specificity

Compared to the study of T cells, the analysis of antibody specificity offers many advantages. Antibodies are highly stable molecules, can be easily handled and stored, and recognize their antigen without helper molecules (e.g. human lymphocyte antigen (HLA) molecules, in the case of T cells). Nevertheless, the identification of MS-specific autoantibodies has turned out to be difficult. It is not clear which compartment is optimal to search for autoantibodies in MS. Serum is readily accessible, but contains the full antibody spectrum of a patient (including those targeting infections) and might not harbor disease-relevant autoantibodies at high concentration. CSF might better reflect the antibody composition in the lesion, but overall contains much less immunoglobulin than serum and is more difficult to access. Antibodies isolated from MS lesions would be optimal for the discovery of autoantibodies, but access to brain biopsy and autopsy material is difficult, and the amount of antibodies extractable from MS lesions is very limited. In one approach to overcoming these problems, antibodies were generated from expanded B-cell clones isolated from the CSF or brain lesions of MS patients.[8, 9] While these recombinant

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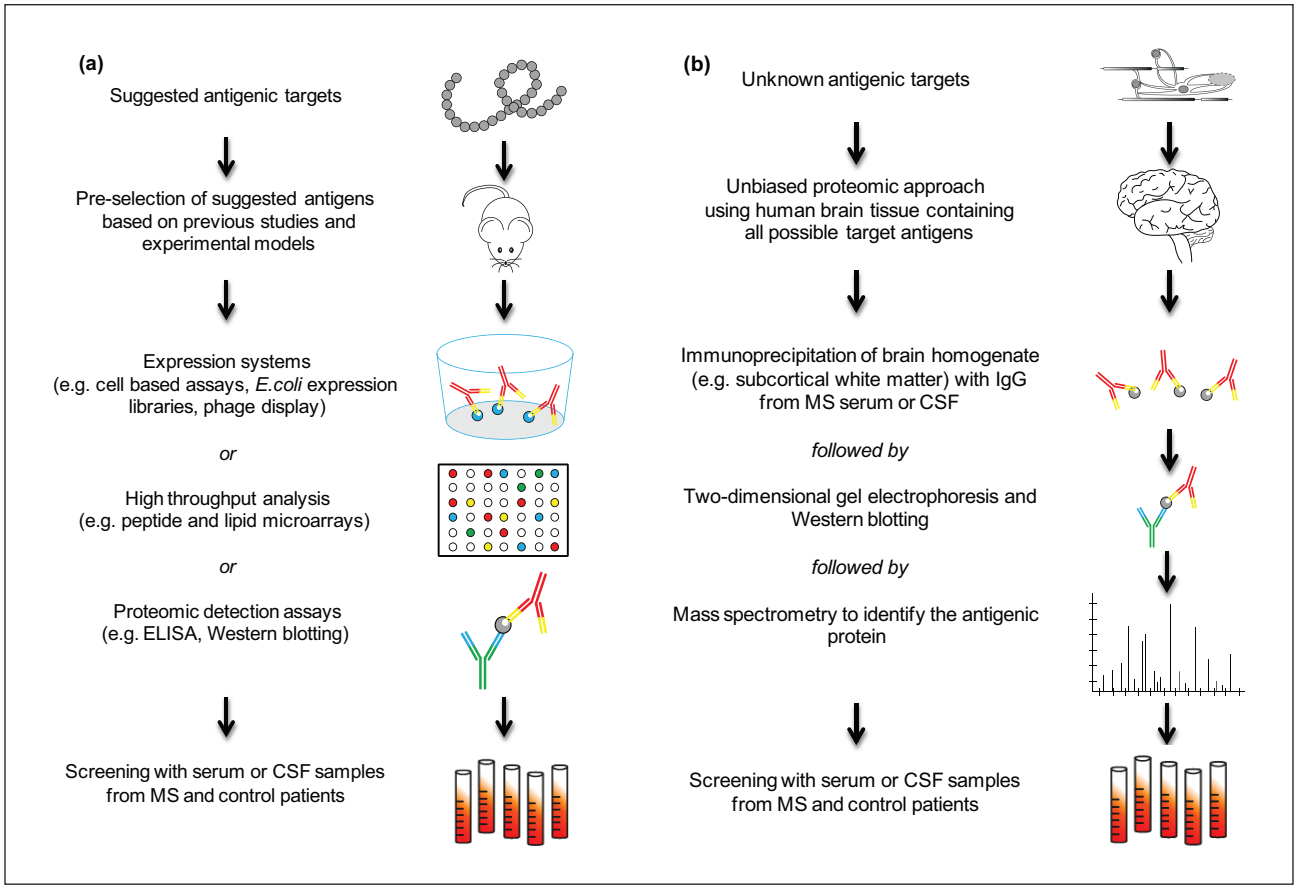

Figure 1. Strategies to identify MS-specific autoantibodies.
CSF: Cerebrospinal fluid; ELISA: enzyme-linked immunosorbent assay; IgG: immunoglobulin G; MS: multiple sclerosis.

molecules represent the antibody secreted by an *in vivo* clonally-expanded B cell, it is unclear whether the particular antibody is centrally involved in the pathogenesis of MS. Moreover, cloning antibodies from the CSF is difficult and time-consuming; therefore, it can only be applied in single or few patients.

Currently, two general strategies are applied to discover autoantibodies in MS. The first strategy is a candidate approach that focuses on one or few candidate proteins suggested by findings from pathological (e.g. expression profile of the protein) or experimental (e.g. encephalitogenicity in experimental animal models) observations. Pre-selected antigens can be purified either from naturally-expressing or transfected cells, or from tissue specimens; and then be used for screening with serum, CSF or cloned antibodies. To overcome the selection bias inherent to such an approach, researchers have introduced novel assays with a broader pre-selection of antigens, by using expression libraries or peptide and lipid microarrays. In the case of expression libraries, the identified protein is determined by the expression clone to which the antibodies are reactive (Figure 1(a)).

The second strategy is an unbiased proteomic approach to screen antibodies from serum, CSF or tissue samples for reactivity with human brain tissue lysates. By this approach, antigens binding to the antibodies can be sequenced to identify the bound targets (Figure 1(b)). Whatever method is used, the identified autoantibody reactivity will have to be confirmed by independent assays (Figure 2).

During the last decade, it has become evident that *in vitro* expression of CNS antigens, especially membrane proteins, is very challenging. Those proteins are usually highly folded and contain post-translational modifications (e.g. glycosylation and nitrosylation) that are often cell specific. Moreover, many membrane proteins are not expressed as monomers, but rather form tetrameric or even oligomeric protein clusters, often in a complex with other proteins. Successful identification of autoantibody reactivity in MS strongly relies on *in vitro* expression systems that can display the protein with all modifications that occur *in vivo*. Although much progress was made with respect to protein expression (e.g. expression of proteins in human neuronal or glial cell lines) and assay systems (e.g. cell-based assays), current *in vitro* expression still does not fully

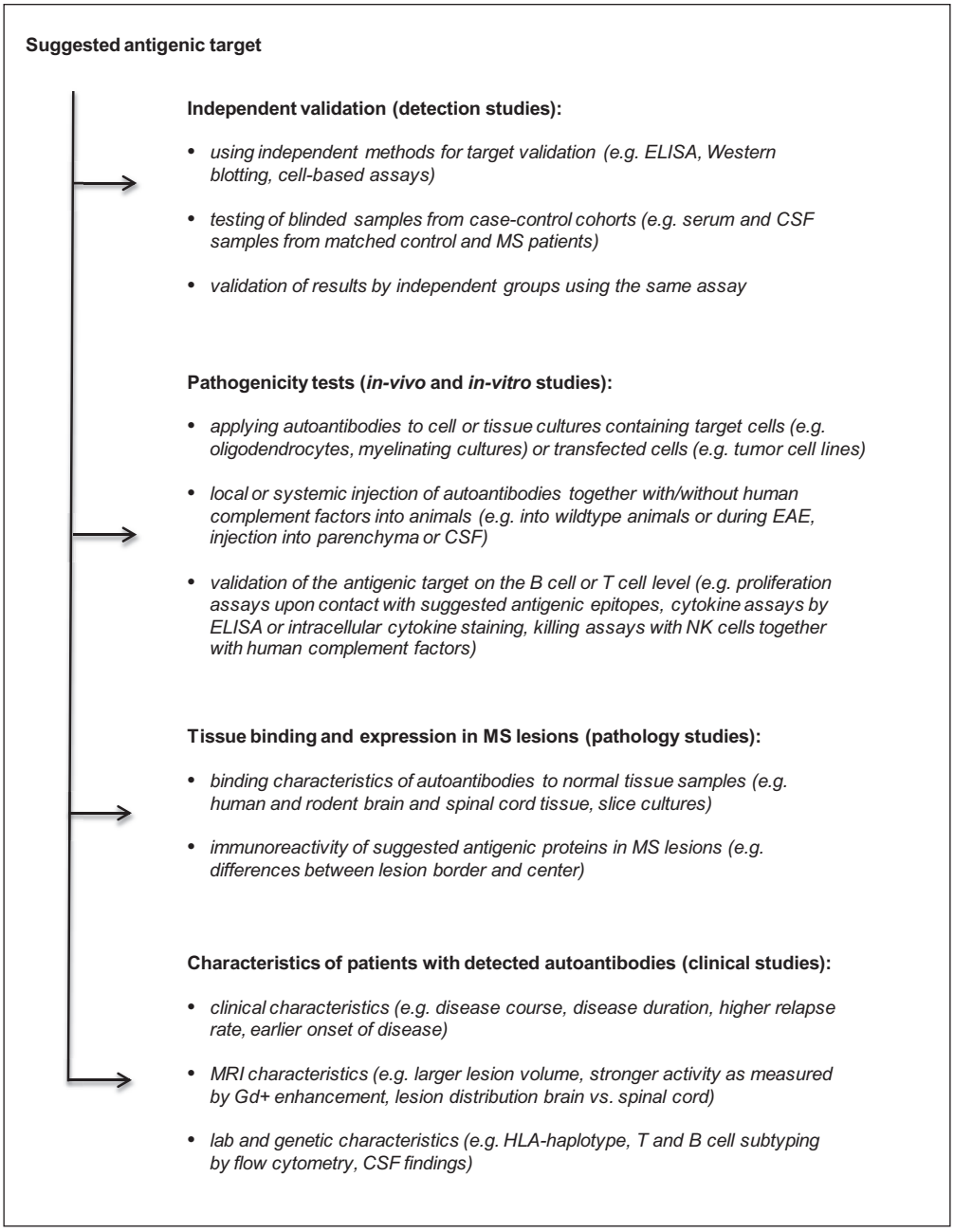

Figure 2. Strategies to validate MS-specific autoantibody reactivity.
CSF: cerebrospinal fluid; EAE: experimental autoimmune encephalomyelitis; ELISA: enzyme-linked immunosorbent assay; Gd: gadolinium; HLA: human lymphocyte antigen; MRI: magnetic resonance imaging; MS: multiple sclerosis; NK: natural killer cells.

display the proteins in the way they are expressed in the human CNS.

Autoantibodies against myelin sheath proteins

Because demyelination is one of the major hallmarks of MS pathology, the traditional focus has been directed towards antibodies targeting proteins of the myelin sheath. Glycoproteins like proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG), myelin-associated oligodendrocyte basic protein (MOBP), myelin-associated glycoprotein (MAG), myelin basic protein (MBP) and oligodendrocyte-specific protein (OSP, claudin-11) are major components of CNS myelin sheaths in humans and are necessary for a proper saltatory conduction along the axons.[10]

In an early study using a solid-phase radioimmunoassay (RIA), a higher antibody response against MAG was found

in MS patients, whereas anti-MBP and anti-PLP antibodies were not enriched in the CSF of MS patients by these methods.[11–13] In contrast, a specific immune response against PLP, but not MBP, could be observed in optic neuritis (ON) patients by applying an immunospot assay.[14] Similarly, conflicting results are reported on the role of MOBP in MS and the occurrence of anti-MOBP antibodies.[15–17] Furthermore, antibodies against both OSP peptides and denatured protein are reported by researchers using Western blotting, a cell-based assay and a microarray analysis; however, there was no specific antibody response against the native protein. Hence, anti-OSP antibodies are rather assumed to be an epiphenomenon in MS.[18,19]

Over the last decade, there has been much focus on MOG as a potential candidate antigen for autoantibodies in MS. In experimental autoimmune encephalomyelitis (EAE), an animal model of MS, anti-myelin antibodies are shown to be necessary for the full expression of the disease, resulting in large demyelinating lesions.[20,21] Initially, studies in MS patients determined the presence of anti-MOG antibodies by enzyme-linked immunosorbent assay (ELISA) or Western blotting, usually with MOG expressed in *E. coli*;[22–24] however, findings of MS-specific anti-MOG antibodies were rather contradictory and refuted in later studies.[25,26] As those initial experiments were performed under denaturating conditions, several groups aimed to establish cell-based assays to demonstrate that antibodies bind to conformational epitopes of the full-length MOG.[27–29]

To increase the sensitivity of anti-MOG detection assays, a MOG tetramer RIA was introduced that is able to detect anti-MOG antibodies to the correctly-folded protein, to a much higher extent.[29] Despite this, high-affinity antibody responses are not consistently observed in the serum of adult MS patients.[27–30] However, in a subgroup of children with a first autoimmune demyelinating event, high titer anti-MOG antibodies are found, especially in those with an onset before the age of 10.[31,32] Whether antibody titers or affinities differ between those children with acute disseminating encephalomyelitis (ADEM) and those with MS has remained controversial. More recently, anti-MOG antibodies were reported in neuromyelitis optica (NMO) patients whom were negative for anti-aquaporin-4 (AQP4) antibodies.[33] Whether MOG antibodies are indeed relevant in NMO is subject to ongoing research.

It was shown that isolated anti-MOG antibodies from MS patients can bind to both mouse and human tissue, *ex vivo*.[27,34] Moreover, anti-MOG antibodies isolated from MS lesions are found to bind to re-folded MOG in *E. coli* and to *in vitro*-translated human MOG.[35] Human anti-MOG antibodies that are injected into EAE animals enhance demyelinating lesions in the CNS, suggesting a functional relevance of these antibodies.[27] Recently, a myelinating culture system was described, to test the demyelinating ability of purified IgG from serum and plasma MS samples, using protein G immunoaffinity chromatography.[36] Although IgG and complement-mediated demyelination is noted in MS by solid-phase immunoabsorption with recombinant MOG protein, Elliott *et al.* suggest that MOG is not a major target of demyelinating antibodies purified from the sera of MS patients.[36]

Autoantibodies against paranodal and axonal proteins

Axonal damage and loss are identified as important pathological features, in addition to demyelination, in the development of MS lesions.[37] In this context, the node of Ranvier has been a particular focus of recent pathological studies, and alterations of para- and juxtanodal proteins have been described.[38–40] Mathey and colleagues[41] used a proteomic screen on a myelin glycoprotein fraction that is enriched from human subcortical white matter by affinity chromatography. Subsequently, by combining two-dimensional (2D) gel electrophoresis, Western blotting and mass spectrometry, they identified Nfasc155 and contactin-2 as potential antibody targets in MS.[41] Autoantibodies against the paranodal 155-kDa isoform of neurofascin (Nfasc155) were detectable by ELISA in the sera of approximately one-third of the MS patients tested. Serum antibodies were also found in a portion of patients with other inflammatory neurological diseases (OIND), but MS patients with a chronic progressive course had higher titers than those of the OIND group, suggesting rather a secondary immune response during disease progression.[41,42]

Recently, antibodies to Nfasc155, along with antibodies to Nfasc186, were also reported in patients with inflammatory peripheral neuropathies, and with combined central and peripheral demyelinating diseases.[43–45] In an animal model, cotransfer of monoclonal antibodies against Nfasc155, together with MOG-specific T cells, resulted in antibody and complement-mediated axonal damage.[41] Anti-contactin-2 antibodies were detectable in the sera and CSF of MS patients; however, there was no difference between patients with MS and other neurological diseases, when the samples were measured by ELISA. Also, the antibody did not induce pathology in animals; however, T cells that are specific for rat TAG1 (rat homologue of contactin-2) could induce inflammatory lesions in the cortical gray matter.[42] Of note, the autoantibodies against contactin-1 have been obtained from chronic inflammatory demyelinating polyneuropathy (CIDP) patients, suggesting that antibodies targeting the structures in the node of Ranvier might be a common feature in demyelinating diseases of the CNS and peripheral nervous system (PNS).[46]

Focusing on axonal integrity, a loss of neurofilaments and in particular of phosphorylated isoforms has been described in MS lesions.[37,47] There are reports of an association between the presence of antibodies against neurofilament light chains and cerebral atrophy in MS patients, although these ELISA results have not yet been independently confirmed.[48,49] Moreover, antibodies against

neurofilaments of 68 kD have been detected by microarray analysis.[17]

Autoantibodies against oligodendrocytic and astrocytic proteins

Beyond the glycoproteins of the myelin sheath and axoglial proteins of the paranodal region, studies implicate target autoantigens at other anatomical sites, including oligodendrocytes and astrocytes, which may go some way toward addressing the limitations of models of MS pathology that are restricted to pure anti-myelin immunity. Notably, most of the studied myelin proteins, in terms of antigens, are only weakly expressed in oligodendrocytic cell bodies and are usually located along axonal fibers. Furthermore, it is known that in the majority of chronic MS lesions, remyelination and development of oligodendrocyte precursor cells (OPCs) fail, due to yet unknown reasons.[50] Hence, autoantibodies targeting myelin-producing oligodendrocytes in particular and OPCs should be considered as important antigenic targets.

Besides oligodendrocytes, attention was recently drawn towards astrocytes and a humoral immune response against the water channel, AQP4. This channel is strongly expressed in the end-feet of astrocytes surrounding blood vessels, enabling a proper water homeostasis in the CNS. Lennon *et al.* identified anti-AQP4 autoantibodies by indirect immunofluorescence of serum samples from patients with NMO.[51,52] The occurrence of a specific antibody response to AQP4 in NMO patients was subsequently confirmed by a number of studies. At present, cell-based assays seem to be the most reliable test to detect and quantify these antibodies.[53,54] Of note, anti-AQP4 antibodies have been shown to have functional relevance, causing a NMO-like pathology if injected into the CNS.[55,56] The identification of the AQP4 antibody has major implications not only for diagnosing NMO, but also for understanding NMO pathogenesis and developing antigen-based treatment strategies.[57]

We investigated the possible targets of serum IgG antibodies in MS by a proteomic approach, based on membrane CNS proteins (Figure 1(b)). Immunoprecipitation of brain homogenate with purified serum IgG from MS patients, followed by 2D electrophoresis and mass spectrometry, revealed the inward-rectifying glial potassium channel KIR4.1 as a potential target of MS autoantibodies.[58] KIR4.1 shares some similarities with AQP4, as both channels are expressed in astrocytes and form complexes, in particular in astrocytic end-feet surrounding blood vessels. In contrast to AQP4, the KIR4.1 channel is also expressed in oligodendrocytes. Interestingly, KIR4.1 is expressed as a homotetramer or heterotetramer with KIR5.1.

We observed that anti-KIR4.1 antibodies preferentially bound to KIR4.1 homotetramer channels that are expressed in oligodendrocytes and astrocytic end-feet (Schirmer *et al.*, submitted). We developed an ELISA with recombinant tetrameric KIR4.1 protein to quantify anti-KIR4.1 antibodies. Serum KIR4.1 antibodies were found in 47% of MS patients and only rarely in healthy donors or patients with other neurological diseases. Similar findings were obtained in children with CIS (clinically-isolated syndrome) or MS.[59] Anti-KIR4.1 antibodies were found to belong to the IgG_1 and IgG_3 isotypes that are known to bind complement and mediate cell lysis. The possible biological activity of these antibodies was suggested by the observation that intracerebral injection of these antibodies leads to a loss of KIR4.1 staining and the activation of the complement cascade. Interestingly, KIR4.1 seems to be lost on oligodendrocytes and astrocytes in acute and chronic active MS lesions (Schirmer *et al.*, submitted). These findings suggest that anti-KIR4.1 autoantibodies differentially target tetrameric structures of antigenic molecules that are specifically located in oligodendrocytes and a subset of astrocytes.

Autoantibody studies with multiple targets or not yet defined targets

A proteomic approach of applying 2D gel electrophoresis and mass spectrometry, without further purification of myelin glycoproteins, was used by Lovato *et al.* to identify a number of proteins, mostly intracellular proteins that are recognized by IgGs from MS patients' sera, among them the oligodendrocytic proteins 2',3'-cyclic-nucleotide 3'-phosphodiesterase type I (CNPase I) and transketolase.[60] Unfortunately, this finding has not been followed up in an independent cohort using a quantifiable assay, although CNPase was implicated as a potential autoantigen by a previous T cell study.[61]

Several groups have applied phage display libraries or *E. coli* expression libraries to identify autoantibodies in CSF or serum samples from MS patients.[62–64] Initial studies used short random peptide phage libraries for screening with CSF IgG; however, this approach revealed MS-specific peptide reactivities that did not lead to any particular protein. Later, a phage display library from an OPC line was screened with CSF samples from MS patients. Archelos *et al.* detected reactivity against oligodendrocytic peptides, and reactive CSF specimens showed binding to OPCs *ex vivo*.[62] Somers and colleagues used cDNA phase display libraries to screen for CSF IgG in MS. Using this approach, they identified a number of targets that were bound by CSF IgG from MS patients, but not controls.[64, 65] The majority of targets were intracellular proteins. Follow-up studies are ongoing to evaluate these targets by conventional assays.[66]

Our group used an *E. coli* expression library including 37,000 proteins from a human brain to search for CSF antibodies in MS. We identified a number of MS-specific protein reactivities targeting intracellular proteins. Interestingly, follow-up studies on these proteins revealed that most of these reactivities target out-of-frame expressed proteins or protein sequences with homology to Epstein Barr virus (EBV) proteins.[67]

Using a microarray assay including 362 myelin and inflammatory-related antigens, Quintana *et al.* could

Table 1. Antigenic targets of autoantibodies in MS.

Target structures	Detection assays	References
Myelin lipids and glycoproteins		
Sulfatides, sphingomyelin, oxidized lipids	MA	Kanter et al., 2006
OSP	WB	Bronstein et al., 1999
	CBA	Aslam et al., 2010
PLP	Immunospot	Sellebjerg et al., 1994
	MA	Quintana et al., 2008 Quintana et al., 2012
MBP	RIA	Panitch et al., 1980
	MA	Quintana et al., 2012
MOBP	TCA	Holz et al., 2000
	MA	Van Haren et al., 2013 Quintana et al., 2012
MAG	RIA	Moller et al., 1989 Johnson et al., 1986
MOG	WB	Lindert et al., 1999 Reindl et al., 1999 Berger et al., 2003 Kuhle et al., 2007 Pelayo et al., 2007
	ELISA	Reindl et al., 1999
	RIA	O'Connor et al., 2005 O'Connor et al., 2007
	MA	Quintana et al., 2012
	CBA	Lalive et al., 2006 Zhou et al., 2006 O'Connor et al., 2007 Brilot et al., 2009 Probstel et al., 2011
Paranodal and axonal proteins		
Nfasc155	2D GE/MS, ELISA	Mathey et al., 2007
Contactin-2	2D GE/MS, ELISA	Mathey et al., 2007 Derfuss et al., 2009
Neurofilament light chains	ELISA	Eikelenboom et al., 2003 Bartos et al., 2007
Neurofilament 68 kD	MA	Quintana et al., 2012
Oligodendrocytic and astrocytic proteins		
KIR4.1 (KCNJ10)	2D GE/MS, ELISA	Srivastava et al., 2012
CNPase	2D GE/MS	Lovato et al., 2008
	TCA	Rosener et al., 1997
	MA	Quintana et al., 2012
Transketolase	2D GE/MS	Lovato et al., 2008
Alu peptides	EL	Archelos et al., 1998
Inflammation-associated and other proteins		
Heat shock proteins:		
HSP60	MA	Quintana et al., 2008 Quintana et al., 2012
HSP70	MA	Quintana et al., 2008 Quintana et al., 2012
CRYAB	MA	Kanter et al., 2006 Ousman et al., 2007 Quintana et al., 2012
RBPJ	MA	Querol et al., 2013
SPAG16	EL	Somers et al., 2008
Coronin-1a	ELISA	Rouwette et al., 2013

CBA: Cell-based assay; CNPase: 2',3'-cyclic-nucleotide 3'-phosphodiesterase; CRYAB: alphaB crystallin; 2D GE/MS: two-dimensional gel electrophoresis and mass spectrometry; EL: phage or *E. coli* expression libraries; ELISA: enzyme-linked immunosorbent assay; HSP: heat shock protein; MA: microarray assay; MAG: myelin-associated glycoprotein; MBP: myelin basic protein; MOBP: myelin-associated oligodendrocyte basic protein; MOG: myelin oligodendrocyte glycoprotein; MS: multiple sclerosis; OSP: oligodendrocyte-specific protein; PLP: proteolipid protein; RIA: radioimmunoassay; RBPJ: recombination signal-binding protein for the immunoglobulin kappa J region; SPAG: sperm associated antigen; TCA: T-cell assay; WB: Western blot.

identify antibody responses in the serum and CSF against several myelin proteins and heat shock proteins (HSPs), in particular PLP and HSP70, which are unique to different disease courses in MS patients.[68] Interestingly, they found in relapsing–remitting MS (RRMS), as compared to primary and secondary progressive MS (PPMS and SPMS, respectively), higher antibody responses against inflammatory-related proteins like HSP60 and HSP70.[68] This group could confirm the presence of antibodies against myelin proteins and HSPs in matched serum and CSF samples of RRMS patients in a later study.[17]

Applying a similar high-throughput microarray analysis consisting of 9,393 possible antigens, Querol *et al.* recently reported higher reacitivites against the recombination signal-binding protein for the immunoglobulin kappa J region (RBPJ) in the CSF of MS patients.[69] Independent validation of the targets revealed a higher antibody response in the CSF of MS patients, compared to controls; however, RBPJ is ubiquitously expressed, and serum antibodies to RBPJ are also observed in other diseases (e.g. cancer).[70] By using microarrays, other groups could detect antibodies against the lipids of the myelin sheath (e.g. sulfatide, sphingomyelin and oxidized lipids) and αB-crystallin (CRYAB), a member of the HSPs, in the CSF of MS patients;[68,71,72] however, antibodies against CRYAB were of low affinity in the CSF.[68,72]

Conclusion

Despite ongoing efforts and continuous improvement in screening and validation methods, no MS-specific autoantibody has been established and broadly validated until today. While there is indirect evidence for the existence of specific and pathophysiologically relevant autoantibodies in MS, the identification of such autoantibodies has turned out to be more difficult than initially anticipated (Table 1). The difficulties in building and preserving the complexity of human CNS proteins in detection assays, particularly of those antigens that are expressed on the cell membrane, and the problems in selecting the right antibodies as bait are most likely responsible for the lack of major progress during the last decade. Nevertheless, recent studies propose interesting targets that are currently undergoing the evaluation and validation process (Table 1 and Figure 2). With the development of more sophisticated screening methods and expression systems that better reflect the structure of autoantigens expressed in the CNS, additional candidates will follow.

Establishing MS-specific autoantibodies would be a major step forward, by providing clues to the targets of the immune response in MS. If these antibodies are pathophysiological and pathogenically relevant, as demonstrated by *in vivo* or *in vitro* models, they will pave the way for specific immune intervention strategies in MS.[53,58] In this context, evidence for specific T-cell responses against identified antigenic epitopes that differ between patients and controls might support the relevance of an identified autoantibody reactivity.[42,55,56,67] Moreover, specific autoantibodies might be of relevance for diagnostic purposes, and for stratifying patients with respect to their prognosis and treatment response. These opportunities should accelerate our efforts to both search for new and validate the upcoming autoantibodies in MS.

Conflict of interest

A patent was filed by RS and BH for the detection of antibodies against KIR4.1, in a subpopulation of MS patients.

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