Multiplexing approaches for autoantibody profiling in multiple sclerosis

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**Abstract**

The preliminary positive effects of B cell depletion therapy in multiple sclerosis (MS) have renewed interest in a potential role of B cells and autoantibodies in the MS disease process. Regardless of a possible pathogenic role of the humoral immune response in MS, the analysis of autoantibodies as disease markers is valuable. Despite intense research, there is no known MS-associated antibody specificity that can individually discriminate between MS patients and controls. Due to the overlap in autoantibody profiles in autoimmune diseases, and due to the complexity of MS, multiplex autoantibody profiling approaches are needed to generate a panel of MS-associated autoantibodies with high combined sensitivity and specificity for MS. In recent years, several multiplexing approaches have been applied in MS autoantibody profiling with promising results regarding the generation of a so-called MS-specific autoantibody fingerprint. We also recently applied a high-throughput autoantibody profiling technique for MS cerebrospinal fluid resulting in the identification of a novel panel of 8 antigenic targets with 45% sensitivity and 86% specificity for the disease.

Identification of MS-specific autoantibody specificities is important for the development of diagnostic and prognostic markers for MS. Moreover, it can provide more knowledge regarding underlying MS disease processes and novel therapeutic targets.

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**Keywords:** Multiple sclerosis, Autoantibody profiling, Multiplexing, Markers, Phage display

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1. **The role of B cells and autoantibodies in multiple sclerosis**

In multiple sclerosis (MS) research, the role of B cells and autoantibodies in the pathogenic disease process has long been a point of debate. The evidence for a primary role of an
abnormal humoral immune response in the MS pathogenesis has up till now been mainly circumstantial [reviewed in \[1,2\]]. The most consistent laboratory abnormality in MS patients is the presence of an intrathecal, antigen-driven immunoglobulin (Ig) production which is demonstrated by oligoclonal immunoglobulin bands in the cerebrospinal fluid (CSF) of more than 90% of MS patients \[3\]. In addition, B cells, plasma cells and myelin-binding antibodies have been reported in MS lesions \[1,2\] and ectopic lymphoid follicles containing proliferating B cells and plasmacells have recently been demonstrated in the meninges of a subset of MS patients \[4\]. Moreover, the presence of a distorted humoral immune response in MS, represented by a higher number of CSF oligoclonal bands and increased CSF Ig levels, has shown to be associated with a worse disease outcome \[5\]. These findings however, do not fully support a causative role for the humoral autoimmune response in the disease. Recently, more conclusive evidence for a role of the humoral immune response in MS is provided by the efficiency of a novel treatment strategy; the preliminary positive results of B cell targeting therapy in a group of MS patients point towards a causal and pathogenic role of the humoral immune response in MS and has led to a renewed interest in B cells and auto-antibodies \[6\]. Even if B cells and autoantibodies do not have a contributing role in the immunopathogenic processes of the disease itself and even if the described abnormalities in the humoral immune system are epiphenomena of the disease, these distortions can contain information regarding the underlying disease processes and can be used for the development of markers for the disease.

2. Autoantibody markers in MS

In the past, different experimental approaches have been applied for the analysis of autoantibody specificities in the serum and CSF of MS patients [reviewed in \[7,8\]]. Table 1 contains a summary of all previously reported antigenic targets of autoantibodies in MS. As it is thought that in MS, the myelin in the central nervous system (CNS) is the primary target of the aberrant autoimmune response, different myelin components were firstly proposed as autoantigen suspects (Table 1). Numerous reports are available on antibodies directed against myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), proteolipid protein (PLP) and myelin-associated glycoprotein (MAG) in MS patients \[7\]. A wide range of anti-myelin antibody sensitivities and specificities for MS have been reported but up till now, none of the myelin-associated candidate antigens definitely discriminates between the humoral immune response in MS patients and healthy controls \[7\]. The true relevance of anti-myelin antibodies in the MS disease process and in the development of MS disease markers remains inconclusive.

Moreover, other CNS-specific components such as neuronal, axonal and glial compounds have been analyzed as autoantibody targets in MS (Table 1) \[8\]. There are however, also reports about the presence of antibodies against more ubiquitous self-antigens such as heat shock proteins and DNA (Table 1) \[7\]. Besides antibodies directed against self components, antibodies against different viruses and bacteria such as Epstein–Barr virus (EBV) and human herpes virus type 6 have also been detected in higher frequencies in MS patients \[7\].

Up till now however, the reported sensitivities and specificities of the identified individual autoantibodies for MS are at best only moderate. From a biomarker point of view, this means that none of the reported antigen–antibody systems can be efficiently used as an individual marker for the disease. To overcome the reported relatively low sensitivities of the individual antibody–specificities in MS, different autoantibody markers need to be combined into a biomarker panel, indicating the requirement for the application of multiplex autoantibody profiling techniques.

3. Multiplexing approaches for autoantibody profiling

In recent years, the need for multiplex autoantibody profiling approaches has become evident within the research domain of autoimmunity [9,10], since autoimmune diseases are characterized by broad autoantibody profiles with considerable overlap between the different diseases [11]. For multiple sclerosis, the necessity for a panel of several markers

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Autoantibody targets in multiple sclerosis.</th>
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<tbody>
<tr>
<td><strong>Myelin antigens</strong> (proteins, glycoproteins and lipids)</td>
<td><strong>Oligodendrocyte antigens</strong></td>
</tr>
<tr>
<td>Myelin oligodendrocyte glycoprotein (MOG)</td>
<td>Surface antigen on oligodendrocyte precursor derived cell line</td>
</tr>
<tr>
<td>Myelin basic protein (MBP)</td>
<td>Oligodendrocyte progenitor cell-specific surface glycoprotein (AN2)</td>
</tr>
<tr>
<td>Myelin associated glycoprotein (MAG)</td>
<td>Oligodendrocyte-specific protein (OSP)</td>
</tr>
<tr>
<td>Proteolipid protein (PLP)</td>
<td>Transaldolase (TAL)</td>
</tr>
<tr>
<td>CSF114/Glc</td>
<td>2′,3′-cyclic nucleotide 3′ phosphodiesterase (CNPase)</td>
</tr>
<tr>
<td>Gc(alpha1,4)Glc(alpha)</td>
<td><strong>Heat shock proteins</strong></td>
</tr>
<tr>
<td>Myelin lipids (phosphatidyldicholine)</td>
<td>Alpha B crystalline</td>
</tr>
<tr>
<td>Galactocerebroside (GalC)</td>
<td>Hsp60</td>
</tr>
<tr>
<td>Cerebellar soluble lectin (CSL)</td>
<td>Hsp70</td>
</tr>
<tr>
<td><strong>Axonal and neuronal antigens</strong></td>
<td>Hsp90β</td>
</tr>
<tr>
<td>Neurofilaments (light and medium chain)</td>
<td><strong>Ubiquitous and other antigens</strong></td>
</tr>
<tr>
<td>Gangliosides (GD1a, GM3, sulfatide)</td>
<td>Proteasome</td>
</tr>
<tr>
<td>Axolemma-enriched fraction (AEF)</td>
<td>DNA</td>
</tr>
<tr>
<td>Tubulin</td>
<td>Heterogeneous nuclear ribonucleoprotein B1 (hnRNP B1)</td>
</tr>
<tr>
<td>Nogo-A</td>
<td>Retinal arrestin</td>
</tr>
<tr>
<td>Nogo receptor (NGR)</td>
<td>Based on [7,8,34–40].</td>
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is also explained by the enormous heterogeneity which is characteristic for the disease and is reflected in the diversity of MS disease courses, treatment efficiencies and prevailing pathogenic processes. Different multiplexing approaches have already been used for the identification of a MS-specific autoantibody fingerprint in MS serum and MS CSF (Fig. 1).

3.1. Antigen arrays

In 2006, Kanter et al. were the first to apply antigen microarrays for autoantibody profiling in MS CSF [12]. Microarrays were composed of 50 distinct brain, myelin and microbial lipids and glycolipids that represented potential targets of the MS autoimmune response including ganglioside, sulfatide, cerebroside, and sphingomyelin. Lipid-specific antibody responses in CSF were compared between 16 MS patients and 11 patients with other neurological diseases. Increased lipid-specific antibody reactivities against myelin lipids including sulfatide, sphingomyelin, oxidized lipids and microbial lipids were shown in individuals with MS [12].

Quintana et al. recently reported the use of an array composed of 362 myelin and inflammation-related antigens (proteins and lipids) that encompassed CNS antigens associated with MS, CNS antigens associated with other neurological diseases and heat shock proteins [13]. Unique serum autoantibody patterns that distinguished relapsing-remitting (RRMS), secondary progressive (SPMS) and primary progressive (PPMS) MS from each other and from healthy controls and other neurological and autoimmune diseases were detected. Moreover, patients with different immunopathologic patterns of MS (based on brain biopsy analysis) could be discriminated based on unique autoantibody profiles [13]. Despite the obtained interesting results, the main drawback of antigen array screening is the fact that it is a so-called biased autoantibody profiling approach. This means that in antigen microarrays, only a small number of known and characterized antigens are spotted as candidate antigenic targets which is not fully representative for the heterogeneity present within the in vivo target tissue in MS, namely the inflamed central nervous system. Moreover, by applying this approach, the identification of novel antigenic targets is not possible (Fig. 1).

An alternative, less biased protein array system for autoantibody profiling in MS was applied by the research group of Hemmer [14]. Protein arrays were prepared by the spotting of 37,000 different expression clones, obtained from a human fetal brain cdNA library. These arrays were probed with individual CSF samples from MS and control patients (patients with other inflammatory and non-inflammatory neurological diseases) to identify the expression clones with strong reactivity in MS patients but not in controls. The most frequent MS-specific reactivities corresponded to EBV peptide sequences. Immunoreactivities towards the identified EBV proteins were significantly higher in the serum and CSF of MS patients compared to controls [14].

3.2. Immunoblotting

Another high-throughput multiplexing approach for autoantibody profiling is the one performed by Lefranc et al. [15]. This research group applied 1-D immunoblotting to compare serum IgG antibody repertoires against human brain homogenates between 82 MS patients and 46 control subjects. Despite the high degree of heterogeneity with regard to number and nature of recognised protein bands, serum IgG antibody repertoires were identified which could distinguish MS patients from controls. Furthermore, specific IgG reactivity profiles that could discriminate between the three forms of MS (RR, SP and PP) were detected [15].

An analogous 1-D immunoblotting procedure of human brain lysates with purified MS CSF IgG was applied by Kolln et al. [16]. Triosephosphate isomerase (TPI) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were identified as MS CSF antibody targets. ELISA screening of CSF samples from clinically isolated syndrome patients, MS patients and patients with other neurological diseases demonstrated that GAPDH-reactive antibodies and the coexistence of TPI- and GAPDH-reactive antibodies were primarily found in MS CSF. Both antigens were proposed as candidate targets for the MS-associated autoimmune response to neurons and axons [16].

The application of 1-D immunoblotting of brain homogenates for autoantibody profiling allows the identification of novel antigenic targets. In addition, the in vivo antigenic composition of the diseased tissue is conserved to a higher extent in this immunoblotting procedure. However, the inherent bias in antigen representation due to use of denaturing polyacrylamide gel electrophoresis (SDS-PAGE) have to be taken into account (Fig. 1).

3.3. Lambda phage expression library screening

Archelos et al. applied an unbiased high-throughput procedure in which a lambda phage cdNA expression library constructed from an oligodendrocyte precursor cell line was screened with pooled CSF from 54 MS patients [17]. By comparison with pooled CSF from patients with other neurological diseases, 6 clones with MS-associated immunoreactivity were identified. For 5 of these clones, a common sequence of 7 amino acids was detected, which was homologous to a translated consensus Alu repeat epitope. By screening sera and CSF from MS patients, it was shown that 44% of the patients reacted with Alu-peptides. Immunostainings with these antibody-positive samples showed strong staining of cytoplasm of oligodendrocyte precursors which indicated the existence of an unknown oligodendrocyte precursor-derived epitope targeted by B cells in a subgroup of MS patients [17].

Owens et al. reported the screening of 2 lambda phage cdNA expression libraries constructed from chronic MS plaques with both crude pooled MS CSF and IgG extracted from pooled MS CSF and MS serum [18]. In this study, no MS-specific target antigens could be discerned. Moreover, screening of a subtracted cdNA library (subtracted with normal human brain), enriched for rare transcripts and sequences over-expressed in MS tissue, with MS CSF did not result in identification of MS-specific autoantibody targets [18].

4. Filamentous phage display for autoantibody profiling

In the 1980s a high-throughput molecular technique, phage display, was developed to identify protein or peptide ligands for a wide range of target molecules [19]. The phage display technique is based on the potential of a filamentous phage virus particle to incorporate foreign DNA into its genome coupled to a
Fig. 1. Multiplexing approaches for autoantibody profiling: a) phage display library screening, b) lambda phage expression library screening, c) immunoblotting, d) antigen arrays. Advantages and disadvantages of the different multiplex autoantibody profiling techniques are summarized. The serological antigen selection procedure entails subsequent rounds of affinity selection of a cDNA phage display library with patient immunoglobulins. An affinity selection round is initiated by incubating phage displaying the cDNA library with pooled patient body fluids (1). During this incubation step, antigen–antibody complexes are formed between the antibodies present in the patient body fluids and their respective target antigens at the surface of the phage (2). These complexes are captured on a solid support by anti-human IgG capture antibody (3) while non-bound phage are washed away (4). Bound phage are eluted (5), amplified through infection of host bacteria (6) and used as input in a subsequent affinity selection procedure (7). The succession of affinity selection and amplification of selected phage results in enrichment of phage-displayed antigens targeted by patient IgG.
gene encoding a phage coat protein. This results in the display of the corresponding DNA product on the phage surface, coupled to the phage coat protein. The strength of the technology lies within the physical link between genotype (DNA) and phenotype (DNA product attached to the viral protein coat), which enables the success of affinity selection and amplification of selected phage particles resulting in powerful enrichment of selected phage (Fig. 1) [19]. In comparison, by the absence of a physical link between genotype and phenotype in the above-mentioned studies based on lambda phage expression library screening [17,18], an enrichment of selected phage is not achievable (Fig. 1). Phage display is not only applicable for the identification of a ligand for one particular target molecule, it can also be used to identify the epitopes that are recognized by polyclonal mixtures of antibodies in healthy or diseased body fluids. A commonly used phage display procedure encompasses the display of synthetically produced peptide libraries [20]. This peptide phage display technique has been used previously for the elucidation of MS autoantibody profiles.

The technique was applied by Cortese et al. to identify peptides and peptide motifs that were recognized by antibodies in the CSF from MS patients [21,22]. The authors showed that selection with individual MS CSF led to the identification of phage-displayed epitopes that rarely reacted with CSF from other MS patients, suggesting that the repertoire of CSF antibodies in MS patients is patient-specific. Furthermore, antibodies against the selected epitopes were demonstrated with a similar frequency in MS sera as compared to control sera, indicating that the target epitopes mimicked ubiquitous antigens to which many individuals are exposed [21]. In a new attempt to identify MS-specific epitopes, 20 different MS CSF samples were pooled for selection of a random peptide library. However, by performing this experiment, the earlier findings of patient-specific antibody reactivities was confirmed. The authors concluded that common antibody specificities are absent in MS patients, or that they are present in very low concentrations [22]. These findings are however contradictory to the results obtained by other research groups that applied peptide phage display for autoantibody profiling in MS.

Dybwad et al. analyzed the CSF antibody specificities within one oligoclonal band from a MS patient using 6-mer, 9-mer and 15-mer random peptide libraries displayed at the surface of filamentous phage [23]. Different distinct but structurally related peptide motifs were detected, which showed significant linear homology with collagen proteins, the 68 kDa neurofilament protein, versican and other proteins from viruses such as human cytomegalovirus, herpes simplex virus and human papillomavirus [23].

Jolivet-Reynaud et al. reported the use of a decapptide phage display library to analyze the antibody specificities in the CSF and serum from 4 MS patients [24]. The peptides that were identified by selections with MS serum were not disease-specific. Selections performed with CSF on the other hand, led to the identification of several motifs which were not detected in serum-selected peptides and were recognized more specifically in MS CSF compared to CSF from patients with other neurological diseases. The combination of the selected MS CSF epitopes allowed the detection of antibodies in 21 out of 60 tested MS CSF, in comparison to only 2 out of 27 control CSF. Amino acid similarities were detected between the selected peptides and envelope regions of multiple sclerosis-associated retrovirus and the related endogenous retrovirus (HERV-W) [24].

Rand et al. have used individual CSF from 14 MS patients for the screening of a hexamer peptide phage display library [25]. CSF from 5 out of 14 MS patients selected a common motif which was found in EBV protein EBNA-1 (Epstein–Barr nuclear antigen 1), alpha B crystalline and in the 65 kDa non-structural cytomegalo virus antigen. It was shown that 60–65% of EBV-seropositive MS patients presented with antibodies against the selected motif, and EBNA-1 associated oligoclonal bands were detected in patients with MS [25].

The main disadvantage of the peptide phage display system is the identification of so-called mimotopes. Mimotopes are peptides forming epitopes that structurally mimic the actual in vivo antigens. This implies that a mimotope and the corresponding actual antigen are not necessarily identical or similar at amino acid level, although sequence similarity cannot be excluded. A labour-intensive characterization of the identified epitope is required for the identification of the actual in vivo target antigen of the detected autoantibody [26]. This drawback of peptide phage display can be overcome by the application of cDNA phage display [27].

5. cDNA phage display for autoantibody profiling in MS

cDNA phage display entails the display of an entire cDNA expression library allowing interaction between cDNA encoded peptides and proteins exposed on the surface of phage particles and antibody selectors. If a cDNA expression library derived from diseased tissue is used for phage library construction, the entire heterogeneity of known and novel expressed antigens present within the affected tissue can be displayed and potentially selected. The antigenic complexity held within the diseased tissue is far greater than that from normal tissue, due to the diversity of processes occurring at this site of autoimmune attack such as inflammatory cell infiltration, activation and migration as well as destruction and possible repair of endogenous brain tissue cells. Moreover, compared to antigen arrays, as yet unknown or uncharacterized expressed sequences are included in the antigenic diversity.

To analyze autoantibody specificities in the CSF of MS patients, we have recently applied a procedure called serological antigen selection (SAS), which is based on cDNA phage display. SAS is a powerful molecular approach which has already been successfully applied for autoantibody profiling in rheumatoid arthritis (Somers K et al., submitted), atherosclerosis [28] and colorectal cancer [29]. Application of the procedure for MS CSF entailed filamentous phage display of a normalized expression library made from three active chronic MS plaques with varying degrees of demyelination and inflammatory activity, obtained from one MS patient [30]. Sequencing of the MS cDNA library confirmed the value of the use of a cDNA expression library made from diseased tissue for the identification of autoantibody targets in MS [30]. The percentage of novel genes and the number of expressed sequences were significantly higher in the MS library compared to normal human brain libraries. The detected sequences were associated with the diversity of processes occurring in and around MS lesions such as inflammatory cell infiltration, activation and migration,
antigen presentation and tissue damage [30]. The MS plaque expression library was cloned C-terminally of filamentous phage coat protein pVI, allowing the expression of full-length cDNA fragments with inherent stop codons [31,32]. Through fusions with minor coat protein pVI, display of cDNA products is monovalent (one cDNA product per phage particle), allowing high-affinity interactions with antibody selectors. The selection of high-affinity interactions is also mediated by the fact that the binding of antibodies to the phage-displayed cDNA products is performed in a solution phase [32].

By application of affinity selection of a MS cDNA phage display library with pooled CSF from 10 RRMS patients (Fig. 1) we recently identified a panel of 4 MS-specific and 4 MS-associated antigenic targets [33]. Immunoreactivity against this panel of 8 novel MS-candidate antigens was analyzed in 73 MS patients, 64 patients with non-inflammatory neurological diseases and 30 patients with other inflammatory neurological diseases. The sensitivity and specificity values of the detection of antibodies against this panel of 8 autoantibody targets for MS are 45% and 86%, respectively. Moreover, antibody reactivity against these 8 targets could be demonstrated in some, but not all of the sera of patients which were antibody-positive based on CSF analysis [33]. Future analysis of the obtained sensitivities and specificities of a combination of these antigen–antibody systems with earlier described autoantibody specificities (such as anti-MBP or anti-MOG antibodies) is warranted to fully elucidate the diagnostic potential of the identified antigen candidates. Moreover, the identification of these novel targets of the humoral autoimmune response in MS will inevitably result in more valuable knowledge regarding the underlying etiology and disease process of MS.

6. Conclusions

Previously, the search for autoantibody targets in MS has focused on preselected, individual target antigen candidates such as different myelin components. Such a restricted view has not led to the discovery of antigenic targets for which immunoreactivity fully discriminates MS patients from controls. Analysis of the entire MS autoantibody profile however, facilitated by multiplexing approaches, has the potential of generating a panel of different antigenic targets with high combined sensitivity and specificity for the disease. In recent years, different multiplexing approaches have been applied for the analysis of the MS autoantibody profile. Several studies reported the identification of distinct serum autoantibody profiles that were able to distinguish MS patients from controls, indicating the value of multiplexing applications. Not all studies were successful at identifying MS-associated antibodies. An explanation for the difference in procedure efficiency can be found in the different applied methodologies and the well-characterized heterogeneity of MS (and thus also the possible heterogeneity of the autoantibody profiles). The combination of different multiplex autoantibody profiling procedures, each with their inherent biases and advantages, is essential to fully capture the autoantibody complexity in MS body fluids.

The identification of a MS-specific autoantibody profile is valuable from a diagnostic biomarker point of view. In addition, the discovery of novel targets of the humoral autoimmune response in MS patients will generate valuable knowledge regarding underlying disease processes and etiology of MS, and can lead to the identification of needed, novel therapeutic targets.

Take-home messages

• Due to the preliminary positive effect of B cell depletion therapy in MS patients, there is renewed interest in the role of autoreactive B cells and autoantibodies in the disease process of multiple sclerosis.
• Despite intense research into the autoantibody reactivities in MS, there is no known antibody specificity which can definitely distinguish MS patients from controls.
• Due to a great overlap in autoantibody reactivities in different autoimmune diseases, multiplexing is essential to generate a broad panel of different autoantibodies with high combined sensitivity and specificity for multiple sclerosis.
• Autoantibody profiling by affinity selecting a MS cDNA phage display library with pooled MS CSF is an unbiased, high-throughput procedure selecting for high-affinity antigen–antibody interactions.
• The identification of autoantibody specificities which are specific for MS will lead to advances in the development of diagnostic and prognostic markers, and can provide important clues regarding underlying disease processes and possible novel therapeutic targets.

Acknowledgments

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C-reactive protein and anti-CCP in systemic lupus erythematosus

High levels of C-reactive protein have been associated with infections, serositis, and severe arthritis in patients with systemic lupus erythematosus. Amezcua-Guerra et al. (Inflammation research 2008;57:555–7) have performed a descriptive case-series study with the objective to identify biomarkers for erosive arthritis in lupus patients. The authors found that C-reactive protein levels were higher in erosive in comparison to non-erosive disease. Interestingly, anti-cyclic citrullinated peptide antibodies (anti-CCP) were observed in patients with erosive arthritis and none of them without this joint alteration. Regarding other biomarkers such as interleukin-6, 4 and 10, and interferon gamma, no difference was noted between the two groups. In non-erosive patients, a negative correlation was seen between interleukin-6 and C-reactive protein. In summary, this study demonstrated that high levels of C-reactive protein and positive anti-CCP may identify systemic lupus erythematosus patients with erosive arthritis.