Autoantibody Profiling in Multiple Sclerosis Reveals Novel Antigenic Candidates

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Autoantibody Profiling in Multiple Sclerosis Reveals Novel Antigenic Candidates

Veerle Somers, Cindy Govarts, Klaartje Somers, Raymond Hupperts, Rob Medaer, and Piet Stinissen

An important contribution of B cells and autoantibodies has been demonstrated in the pathogenesis of multiple sclerosis (MS), leading to interest in the use of such autoantibodies as diagnostic or prognostic biomarkers. The objective of this study was to identify novel Ab biomarkers for MS using "serological Ag selection". Using a phage display library derived from MS brain plaques, we applied serological Ag selection to identify antigenic targets specifically interacting with Abs present in the cerebrospinal fluid (CSF) of 10 relapsing-remitting MS patients. These antigenic targets were further evaluated on a large panel of CSF from 63 other MS patients, 30 patients with other inflammatory disorders, and 64 patients with noninflammatory neurological disorders. A panel of eight antigenic targets was identified that showed a 86% specificity and 45% sensitivity in discriminating MS patients and controls. Four of the antigenic targets showed exclusive reactivity (100% specificity; 23% sensitivity) in the MS group as compared with the control group. Detailed bio-informatic analyses revealed a novel Ag, SPAG16. Among the novel phage peptides identified, novel epitopes were generated from untranslated sequences and out-of-frame sequences. Of 10 relapsing-remitting patients used for serological Ag selection, Ab reactivity toward one of the eight antigenic targets was also demonstrated in serum of 38% CSF-positive patients. Autoantibody profiles against epitopes derived from MS brain tissue could serve as diagnostic markers or form the basis for the identification of a subgroup of MS patients. The Journal of Immunology, 2008, 180: 3957–3963.

Multiple sclerosis (MS) is a complex disease represented by several pathophysiological processes. Previous studies have revealed the heterogeneity of the disease, displayed by different clinical disease courses and different responses to therapy, as well as the existence of four pathogenetic mechanisms of demyelination (1–3). One of these pathogenetic subtypes is characterized neuropathologically by Ab-dependent immune mechanisms involved in the formation of MS lesions (1, 4).

In the past, an important role of autoreactive B cells and autoantibodies has been demonstrated (5, 6). Recent studies uniformly showed clonal expansion of Ab-secreting B cells in the CNS and cerebrospinal fluid (CSF) of patients with MS (7–11). Furthermore, detection of oligoclonal Abs in CSF of MS patients is an important diagnostic marker in MS (12, 13). It is generally accepted that a single marker is unlikely to serve as a general diagnostic or prognostic tool to cover the heterogeneity of the disease. Therefore, the development of a panel of biomarkers, specific for different pathophysiological mechanisms, will be crucial for the further understanding of the pathogenesis of MS, as well as for diagnosis, classification, evaluation of disease activity, and theranostic applications.

The search for biomarkers in bodily fluids such as CSF and serum of MS patients has been the focus of many studies. Numerous studies have reported the recognition of CNS myelin autoantigens such as myelin basic protein (MBP), proteolipid protein, myelin oligodendrocyte glycoprotein, and myelin-associated glycoprotein by autoantibodies present in CSF and serum of MS patients, but also in patients with other inflammatory neurological diseases (OIND) and noninflammatory neurological diseases (NIND) as well as healthy controls (14–18). Until now, information on involvement of specific Ags in MS has been limited. More recently, other CNS Ags have been identified that are potential biomarkers in MS (19, 20). The role of these Ags in MS pathogenesis is currently being further studied.

In the present study, we report the identification and initial characterization of autoantibody binding peptides isolated by "serological Ag selection" (SAS) on a phage display library of cDNA products expressed in MS brain plaques. The results obtained were also correlated to disease duration, disability, and different clinical courses of disease. The autoantibody profiles against these selected peptides can be used as candidate biomarkers for the detection of MS.

Materials and Methods

Patients and controls

CSF samples were obtained from 73 MS patients, 30 patients with OIND (e.g., meningitis, polyneuropathy, neurosarcoidosis), and 64 patients with
Cloning of a MS cDNA library for pVI display and SAS of phage pVI-displayed cDNA repertoires

A normalized cDNA library (1.0 × 10⁸ primary recombinants) derived from three active chronic MS plaques from one patient, with varying degrees of demyelination and inflammatory activity (gift from Dr. M. B. Soares, University of Iowa), was used to construct a MS cDNA display library by cloning it as a fusion protein with filamentous phage minor coat protein pVI. Therefore, the library was transferred to our phage display vectors, named pSPVIA, pSPVIB, and pSPVIC, with each encoding one of three reading frames. Details of the cloning procedure are described in Ref. (21).

The SAS procedure was performed as described previously (21). In brief, CSF samples of 10 randomly selected untreated relapsing-remitting (RR)-MS patients were pooled and used for affinity selections. Before the start of the selection procedure, CSF samples were absorbed against Escherichia coli and phage components as described in Ref. (21). Following adsorption, pooled CSF was stored at −80°C. Subsequently, pooled pre-absorbed CSF was used for the selection procedure. Affinity selections were performed as described before (21). In brief, an immunotube (Nunc) was coated with rabbit anti-human IgG (Dako) in coating buffer (0.1 M sodium carbonate (pH 9.6)). After washing the immunotube, the tubes were blocked with 2% milk powder in PBS. For the first round of the selection procedure, phage were prepared from the MS cDNA library cloned in the three phage display vectors pSPVIA, pSPVIB, and pSPVIC. Phage were prepared as described previously (22). Approximately 10⁸ phage were added to pooled preabsorbed CSF (1/5 (v/v) diluted in 4% milk powder in PBS) and incubated on a rotating platform. After washing the coated immunotube, the preincubated CSF and phage mix was transferred to the coated immunotube and incubated on a rotating platform, followed by standing at room temperature. Tubes were then washed extensively to remove nonbinding phage. Binding phage were eluted and neutralized as described before (23). E. coli TG1 cells were infected with output phage and plated on 2xYT agar plates containing ampicillin and glucose (16 g/L bacto-trypetone, 10 g/L yeast extract, 5 g/L NaCl, 15 g/L bacto-agar, ampicillin at 100 µg/ml, and glucose at 2%) at each round of selection. Resultant colonies were scraped and phage were rescued for further rounds of affinity selection. To monitor enrichment of specific clones, input and output phage from each round of selection were titrated and the ratio of output/input phage was determined. After several rounds of selection, individual colonies were selected and the insert size and sequence was determined as described in Ref. (21). Sequences were submitted to GenBank for basic local alignment search tool homology search.

Phage ELISA

ELISA of ligand-displaying phage was performed as described in Ref. (21). Immunoreactivity for each phage peptide (OD sample) was measured in relation to an internal control signal (reactivity against the empty phage, or OD background). A ratio of OD sample/OD background >1.5 was considered positive in the ELISA. CSF samples were diluted 1/5 (v/v) and serum samples 1/100. Most CSF and serum samples tested showed equivalent total serum and CSF IgG concentrations. CSF IgG concentrations >10 mg/dl were diluted to normal reference values. For competition ELISA, CSF was preincubated in the presence of 0–50 nmol/50 µl synthetic peptide UH-CSFP1.1 (ASSRGYEDLRTF) or random peptide. Subsequently, the immunoreactivity to phage UH-CSFP1.1 was determined according to the standard phage ELISA procedure.

Northern blot analysis

Plasmid was isolated using the Qiagen Plasmid Midi Kit (Qiagen) according to the manufacturer’s instructions. The isolated plasmid was EcoRI/NorI-digested and the excised DNA was gel-purified (GFX PCR DNA and gel band purification kit, GE Healthcare).

The excised DNA fragment was used as probe in Northern blot. Probes were labeled with [α-32P] using the High Prime DNA labeling kit (Roche). Briefly, 50 ng excised DNA was first denatured during 10 min in boiling water and immediately chilled on ice. The labeling mix was added to the DNA and after 45 min incubation at 37°C, the reaction was stopped by addition of 0.2 M EDTA. Labeled DNA was purified with Sephadex G75 columns, and radioactivity was measured with a scintillation counter.

Characterization of the enriched phage clones

Among the enriched clones, a total of 52 clones were selected. cDNA inserts were sequenced and the translated protein sequences were determined. Sequence analysis revealed eight antigenic targets, which we annotated with the name UH-CSFP- number, which is short for University Hassanl-CSFP pool-number of the clone. These sequences corresponded to known proteins expressed in the correct reading frame, but also homology to untranslated regions of expressed genes.
Table II. Detailed information on Ags retrieved by the selection of a phage-displayed MS cDNA expression library on MS patient CSF

<table>
<thead>
<tr>
<th>GenBank No.</th>
<th>Identity</th>
<th>Translated Protein Sequencea</th>
<th>Sizeb</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>UH-CSFP1.1</td>
<td>NM014382</td>
<td>HS′ ATP2C1, transcript variant I</td>
<td>12</td>
<td>3′-UTR</td>
</tr>
<tr>
<td>UH-CSFP1.2</td>
<td>NM199478</td>
<td>PLP1, transcript variant 2</td>
<td>23</td>
<td>3′-UTR</td>
</tr>
<tr>
<td>UH-CSFP1.3</td>
<td>BX509701.1</td>
<td>HS DKFZ686A1481</td>
<td>53</td>
<td>Estimate</td>
</tr>
<tr>
<td>UH-CSFP1.4</td>
<td>BC006427</td>
<td>HS KIAA1279</td>
<td>14</td>
<td>Out-of-frame</td>
</tr>
<tr>
<td>UH-CSFP1.5</td>
<td>NM00729</td>
<td>HS PACSIN2</td>
<td>11</td>
<td>3′-UTR</td>
</tr>
<tr>
<td>UH-CSFP1.6</td>
<td>AC114947.2</td>
<td>HS chromosome 5 clone CTD-2636A23</td>
<td>104</td>
<td></td>
</tr>
<tr>
<td>UH-CSFP1.7</td>
<td>BC032450</td>
<td>HS chromosome 10 open reading frame with retained intron</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>UH-CSFP1.8</td>
<td>BC067756.1</td>
<td>HS sperm-associated Ag 16</td>
<td>121</td>
<td>In frame</td>
</tr>
</tbody>
</table>

\[a\] Stop codons are indicated by asterisks (*).
\[b\] Size of protein product in amino acids fused to pVI coat protein.
\[c\] HS, homo sapiens.

such as the 3′ untranslated region sequence (3′ UTR) of proteolipid protein, or homology to out-of-frame sequences were obtained (see Table II). The phage peptides encoded by untranslated regions of expressed genes or out-of-frame sequences were aligned to protein databases in National Center for Biotechnology Information (NCBI) using NCBI BLAST and revealed protein identities that significantly matched peptide sequences (see Table III). The size of the putative CSF-reactive epitopes ranged from 11 to 121 amino acids.

In initial experiments, we assessed the reactivity of the individual MS CSF specimens used for the selection procedure against the eight enriched antigenic peptides. As shown in Table IV, of the 10 CSF samples from RR-MS patients, 8 contained Abs that reacted with at least one phage-peptide clone. These clones were used for subsequent screening on a large panel of CSF from other MS patients as well as CSF from patients with OIND and NIND.

Of the 10 RR-MS patients used for the selection procedure, paired serum samples were available for 8 patients and were used for screening for Ab reactivity toward the eight enriched antigenic peptides. In three of eight patients with Ag-specific Abs present in CSF, reactivity toward one of the eight antigenic cDNAs was also found in paired serum. Quantitative differences did not appear to be due to variations in total CSF/serum IgG, because after normalizing all CSF/serum samples to the same IgG concentration, the same results were obtained as when CSF samples were diluted 1/3 (v/v) and serum samples 1/100. Fig. 1 represents an ELISA screen of the immunoreactivity of paired CSF-serum samples adjusted to the same IgG concentration tested to UH-CSFP1.1. Reactivity toward this clone was found in two MS CSF samples (MS-CSF4 and MS-CSF8) and also in the corresponding sera. For MS patient 8, the signal on CSF tested was much higher than that on serum, which is consistent with the dilution of the Ag-specific Abs present in the serum, when Abs are intrathecally produced. However, for MS patient 4, higher reactivity was found in serum. Additionally, no reactivity in paired serum samples was demonstrated in patients with Ab-negative CSF.

Table III. Protein sequence alignmentsa

<table>
<thead>
<tr>
<th>GenBank No.</th>
<th>Identity</th>
<th>Translated Amino Acid Size</th>
<th>Homology on Amino Acid Levelb (Accession No.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UH-CSFP1.1</td>
<td>NM014382</td>
<td>12</td>
<td>72% (8/11) cerebral protein 1 (O0043159)</td>
</tr>
<tr>
<td>UH-CSFP1.2</td>
<td>NM00729</td>
<td>23</td>
<td>88% (8/9) type XIX collagen a1 chain (BAA23099.1)</td>
</tr>
<tr>
<td>UH-CSFP1.3</td>
<td>NM00729</td>
<td>53</td>
<td>75% (9/12) WASP interactor protein (AAD00898)</td>
</tr>
<tr>
<td>UH-CSFP1.4</td>
<td>NM00729</td>
<td>14</td>
<td>66% (6/9) TRIOBP isoform 3 (ABB59559.1)</td>
</tr>
<tr>
<td>UH-CSFP1.5</td>
<td>NM00729</td>
<td>11</td>
<td>72% (8/11) SLC37A3 protein (AAH05022.1)</td>
</tr>
<tr>
<td>UH-CSFP1.6</td>
<td>NM00729</td>
<td>104</td>
<td>46% (13/28) GRIP1-associated protein 1 isoform 2 (NP_997555.1)</td>
</tr>
<tr>
<td>UH-CSFP1.7</td>
<td>NM00729</td>
<td>55</td>
<td>100% (8/8) KIAA1931 (BAB67824)</td>
</tr>
</tbody>
</table>

\[a\] Phage peptides/proteins corresponding to UTRs of expressed genes or out-of-frame sequences in the coding region of known genes were aligned to protein databases in National Center for Biotechnology Information (NCBI) using NCBI basic local alignment search tool (BLAST). Protein identities are known proteins that significantly match the peptide sequence. All sequences were annotated using BLAST analysis.

\[b\] Significant homology of the peptide to a protein annotation in the public domain. For large open reading frames, homology searches were performed based on searches for nearly exact matches.
Table IV.  Reactivity of panel of eight phage clones on individual MS CSF used for the selection procedure

<table>
<thead>
<tr>
<th>Phage cDNA Clones</th>
<th>CSF&lt;sup&gt;a&lt;/sup&gt;</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>No. Reactive CSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>UH-CSFP1.1</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>2/10</td>
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<tr>
<td>UH-CSFP1.2</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>4/10</td>
</tr>
<tr>
<td>UH-CSFP1.3</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>1/10</td>
</tr>
<tr>
<td>UH-CSFP1.4</td>
<td></td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>3/10</td>
</tr>
<tr>
<td>UH-CSFP1.5</td>
<td></td>
<td>+</td>
<td>+</td>
<td>-</td>
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<td>4/10</td>
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<tr>
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<td></td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>6/10</td>
</tr>
<tr>
<td>UH-CSFP1.7</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>1/10</td>
</tr>
<tr>
<td>No. positive phage cDNA clones</td>
<td>3/8</td>
<td>3/8</td>
<td>0/8</td>
<td>2/8</td>
<td>2/8</td>
<td>0/8</td>
<td>1/8</td>
<td>2/8</td>
<td>3/8</td>
<td>6/8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> +, positive ELISA signal (ratio OD sample/OD background > 1.5); -, negative ELISA signal (ratio OD sample/OD background < 1.5).

Detailed serological analysis of the MS panel

Next, clones were tested on a large panel of individual CSF specimens not used for the selection procedure (n = 63 for MS patients (54 RR-MS, 3 secondary progressive (SP)-MS, and 6 primary progressive (PP)-MS patients), n = 30 for OIND patients, and n = 64 for NIND patients). Characteristics of the study population are shown in Table V. The results of the phage ELISA screening of the individual phage-cDNA clones on 167 different CSF are presented in Table VI. All Ags tested showed exclusive or preferential reactivity in the MS group as compared with the control group. Reactivity to at least one of the clones UH-CSFP1.4–UH-CSFP1.7 was demonstrated in 17 of 73 (23%) MS CSF, whereas no reactivity toward the OIND and NIND CSF specimens was observed. Reactivity to at least one of the remaining clones (UH-CSFP1.1–UH-CSFP1.3 and UH-CSFP1.8) was higher in the MS group (25/73 (34%)) as compared with the control group (13/94 (14%)), and therefore these clones were also defined as clones with a MS-related serological profile.

In total, 33 of 73 (45%) MS patients showed CSF IgG Abs reactive with at least one of the panel of eight antigenic targets. The highest frequency of Ab responses in MS CSF with no reactivity in the control group was found in UH-CSFP1.6. All CSF samples tested showed equivalent total CSF IgG levels. CSF samples with high IgG concentrations were normalized to the normal CSF concentration range.

To determine whether the observed autoantibody signature of MS CSF is due to the MS brain plaque-derived peptides, two MS CSF specimens (one positive (MS-CSF8) and one negative (MS-CSF26) for UH-CSFP1.1) were preincubated with the synthetic peptide UH-CSFP1.1 (ASSRGYEDLRF) representing the cDNA insert of clone UH-CSFP1.1 and with a nonspecific (random) peptide. As shown in Fig. 2, preincubation with UH-CSFP1.1 peptide clearly inhibited the formation of specific IgG Ab/phage UH-CSFP1.1 complexes for MS-CSF8, while no inhibition was found for MS-CSF26. In contrast, CSF reactivity against clone UH-CSFP1.1 was not inhibited by addition of the random peptide.

Finally, we tested whether immunoreactivity against the antigenic targets is persistent. Therefore, a comparative ELISA screening was performed on CSF specimens collected at different time points (1 year after first CSF collection) in some of the patients. At present, we observed the same level of reactivity toward two antigenic targets (UH-CSFP1.1 and UH-CSFP1.2) in CSF collected 1 year after initial CSF collection as compared with the first CSF specimen tested (data not shown).

Expression pattern of novel MS markers

Northern blot analysis of the antigenic targets with no reactivity in the control group was performed on a variety of normal human tissues. UH-CSFP1.4 gave a transcript of 1.9 kb and was highly expressed in brain, heart, and placenta, and to a lower extent in skeletal muscle, kidney, and liver. UH-CSFP1.7 gave a transcript size of 5.1 kb and showed a high expression in brain, heart, and skeletal muscle (Fig. 3). So far, no transcript could be detected for UH-CSFP1.5 and UH-CSFP1.6.

Autoantibody reactivity and clinical data

We next examined whether reactivity to our antigenic panel was associated with a particular disease phenotype. Autoantibody reactivity to at least one of the eight antigenic targets was demonstrated in 30 of 64 (47%) RR-MS patients, 3 of 6 (50%) PP-MS patients, and 0 of 3 SP-MS patients. Demographic variables and Expanded Disability Status Scale (EDSS) scores in Ab-positive and Ab-negative MS patients are shown in Table VII. No differences were observed in age between Ab-positive and Ab-negative patients.
patients. Ab reactivity could be observed in some patients at time of diagnosis and was present in patients with short disease duration (<1 year), but also in patients with a disease duration >10 years. However, no correlation was found between Ab reactivity and disease duration.

To assess the possible correlation between Ab reactivity and disease severity, we examined the relationship between Ab reactivity and EDSS score. Ab reactivity was found in 21 of 50 (42%) of patients with EDSS of <3, 6 of 11 (54%) of patients with EDSS of 3 or 3.5, and 3 of 5 (60%) of MS patients with EDSS of 4. Although a higher percentage of patients showed reactivity to the panel of eight antigenic cDNAs with increasing EDSS score, this difference was not significant.

Discussion

In this study, we used SAS to identify autoantibody biomarkers in patients with multiple sclerosis. Specifically, we constructed a cDNA phage display library in which cDNA products derived from MS brain plaques were expressed as a fusion to minor coat protein pVI of filamentous phage M13. The MS cDNA display library was panned to identify phage cDNA clones that bind autoantibodies in CSF specimens from MS patients. A panel of eight antigenic targets, which showed a 86% specificity and 45% sensitivity in discriminating MS patients and controls, was retrieved. Besides a possible role in the immediate diagnosis of patients suspected of having MS, the antigenic targets might also assist in subtyping MS patients. Additionally, preliminary finding of autoantibody reactivity in serum of some MS patients confirms the diagnostic potential of these markers.

The search for novel autoantibody biomarkers has risen enormously. Previous studies based on phage display approaches were used to analyze Ab specificity in MS patients (24 –27). These studies led to the identification of possible target peptides, but after detailed serological analyses, no differences were found between patients and controls (26, 28, 29). In this study, we combined the use of a library of cDNAs preferentially expressed in MS brain plaques (30) with the strong selective power of the SAS procedure to isolate MS-specific Ags, and we screened these novel targets on a large group of MS patients and controls. To our knowledge, this is the largest panel of antigenic targets identified, which after extensive screening resulted in the identification of Ags with MS specificity. By relying on multiple immunogenic MS-specific antigenic targets, this approach may be an improvement over a single biomarker.

Autoantibody reactivity to at least one of the eight antigenic targets was demonstrated in 45% of MS patients, including RR-MS and PP-MS patients, a finding suggesting that biomarkers are representative for different forms of the disease. To draw conclusions on reactivity in SP-MS patients, screening of a larger group of SP-MS patients is mandatory. The detection of autoantibody reactivity at time of diagnosis, and their presence in serum specimens, demonstrates the diagnostic potential of these biomarkers. Moreover, their persistent presence in CSF underlines their possible relevance as disease markers. Additionally, the finding that a higher, though not significant percentage of patients showed reactivity to the panel of eight antigenic targets with increasing EDSS score is intriguing. This result is in line with a study by Robinson et al., who demonstrated that increased diversity of autoantibody responses in acute experimental encephalomyelitis predicted a more severe clinical course (31). However,

![Figure 2](image-url)

**FIGURE 2.** Solution phase assays demonstrate high affinity and specificity of CSF Abs to UH-CSFP1.1. The UH-CSFP1.1 peptide was preincubated at different dilutions with MS-CSF8 and MS-CSF26, respectively, and subsequently the remaining immunoreactivity was measured by ELISA. Competition by the UH-CSFP1.1 peptide is displayed. No competition was measured with the random peptide.

![Figure 3](image-url)

**FIGURE 3.** Expression profile of novel antigenic targets in normal tissues. Expression patterns are shown for UH-CSFP1.4 and UH-CSFP1.7. The lower panel shows a control hybridization with an actin probe. *Lane 1*: brain; *lane 2*: heart; *lane 3*: skeletal muscle; *lane 4*: colon; *lane 5*: thymus; *lane 6*: spleen; *lane 7*: kidney; *lane 8*: liver; *lane 9*: small intestine; *lane 10*: placenta; *lane 11*: lung.
Table VII. Comparison of Ab-positive and Ab-negative patients with established MS

<table>
<thead>
<tr>
<th></th>
<th>Antibody-Positive (n = 33)</th>
<th>Antibody-Negative (n = 40)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean ± SD (years)</td>
<td>37.7 ± 8.9</td>
<td>39.4 ± 10.0</td>
<td>NS</td>
</tr>
<tr>
<td>Disease duration, mean ± SD (years)</td>
<td>3.6 ± 3.3</td>
<td>4.3 ± 5.2</td>
<td>NS</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>11</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>22</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>EDSS, mean ± SD</td>
<td>2 ± 1</td>
<td>2 ± 1</td>
<td>NS</td>
</tr>
</tbody>
</table>

* Age and disease duration were compared with t tests, and categorical variables were compared by χ² testing with appropriate degrees of freedom.

Further studies are needed to determine the association of Ab reactivity and increasing disease score.

Four of the antigenic targets showed exclusive reactivity in the MS group as compared with the control group. The remaining four Ags were substantially more reactive with CSF from MS patients than with CSF from controls (Table VI). One of these phage clones represents a known protein Homo sapiens sperm-associated Ag 16 (SPAG16). SPAG16 is a PF20 protein homolog, of which five different isoforms exists. Isoform 1 (synonyms PF20 variant 1a) is a sperm-associated WD repeat protein orthologous to Chlamydomonas. The presence of WD repeats places it in a family of proteins known to mediate protein–protein interactions (32, 33). Smith and Lefebvre (34), who localized the PF20 protein at the transverse bridge of the two central microtubules, hypothesized that PF20 is required for central microtubule assembly and/or stability and flagellar motility. In this study, autoantibody reactivity was seen against isoform 4, which is synonymous with PF20 variant 2a. Isoform 4 is detected in brain and testis, and at lower levels in kidney, heart, pancreas, thyroid, ovary, adrenal gland, spinal cord, trachea, and liver. The cDNA clone was identified in a hypothalamic cDNA library, and it encodes a protein product of 183 amino acids (35, 36). This isoform is completely devoided of WD repeats. Smith and Lefebvre (34) suggested that the PF20 amino-terminal half could be involved in the binding to one central microtubule, although this region does not encode a known microtubule-binding domain. According to this hypothesis, the human PF20 isoform 4 may be implicated in complexes with microtubules in a large panel of cells; however, its specific function remains to be elucidated. At present, we have successfully expressed both partial and full-length SPAG16 protein (data not shown), which allows further investigation into the possible pathogenic role of this protein and the role of anti-SPAG16 Abs in MS pathology.

A particular phenomenon demonstrated in this study was the finding of autoantibody reactivity to novel peptide sequences encoded by transcript variants of genes. In general, several alternative splicing patterns have been reported, which include exon skipping or inclusion, the use of alternative 3' splice site exons or 5' splice exons, mutually exclusive exons, or intron retention. Ng and colleagues elegantly showed that increased alternative splicing of autoantigen transcripts provides the structural basis of untolerized epitopes (37). In that study, the extent of alternative splicing within 45 randomly selected self-proteins associated with autoimmune disease was compared with 9554 randomly selected proteins in the human genome by using bioinformatics analysis. Isoform-specific regions that resulted from alternative splicing were studied for their potential to be epitopes for Abs or T cell receptors. The authors demonstrated that alternative splicing occurred in 100% of the autoantigen transcripts, which was significantly higher than the 42% rate of alternative splicing observed in the 9554 randomly selected gene transcripts. Within the isoform-specific regions of the autoantigens, 92 and 88% encoded MHC class I- and class II-restricted T cell Ag epitopes, respectively, and 70% encoded Ab-binding domains. The authors concluded that alternative splicing may be an important mechanism for the generation of untolerized epitopes that may lead to autoimmunity, and that the product of a transcript that does not undergo alternative splicing is unlikely to be a target Ag in autoimmunity. In addition, Yeo and colleagues analyzed variation in alternative splicing across different human tissues (38). This study distinguished the human brain, followed by testis and liver as having unusually high levels of alternative splicing (38). This phenomenon of alternative splicing could explain the finding of autoantibody reactivity against some of our antigenic targets, which contained novel epitopes that encoded alternative splicing such as intron retention by a cDNA clone with retained intron for UH-CSF1.7 and novel isoforms of PACSIN for UH-CSF1.5, as well as SPAG16 for UH-CSF1.8. The most intriguing phenomenon appears to be a region of transcript variant 2 of the proteolipid protein 1, which shows 100% homology to UH-CSFP1.2. Using BepiPred analysis (39), we determined the presence of linear B cell epitopes in the protein sequences identified. Seven of eight antigenic targets contained one or multiple linear B cell epitopes, which could explain autoantibody reactivity toward these epitopes.

Apart from alternative splicing, there are several other mechanisms by which Ags can elicit an immune response. These include gene activation, gene overexpression, viral gene expression, gene mutation, or posttranslational modifications. Expression analysis of UH-CSF1.4 and UH-CSFP1.7 showed that high expression was found in normal brain (Fig. 3). However, a possible overexpression in MS brain could be the mechanism of immunogenicity. Of note, three of the eight clones represented epitopes similar to viral sequences such as BFRA1 (EBV) (see Table III). Cepok et al. recently reported the identification of peptide sequences derived from EBV proteins as putative targets in MS (40). In the present study, similarity to other EBV epitopes was obtained. The exact mechanism that leads to immunogenicity of each of the antigenic cDNAs remains to be elucidated.

Overall, all of the antigenic cDNAs identified showed epitopes that could be relevant to the disease process. Interesting, several clones represented epitopes encoded by cerebral proteins, or epitopes from cDNA sequences originating from brain libraries (see Table III).

Another explanation for the high homology to known cDNAs, but weak homology on the protein level, could be the occurrence of mimotopes. These represent stretches of amino acids that mimic an Ag but are not homologous at the sequence level. Autoantibody reactivity toward these mimotopes has been demonstrated by several studies (40, 41). Additionally, of both known and novel protein products identified, we and others succeeded in correct expression of the protein products (40). Further characterizations of these markers using other experimental platforms are under way.

In conclusion, we have described the use of the SAS procedure for the discovery of autoantibody biomarkers in MS. For the first time, we report the identification of a panel of antigenic targets specifically interacting with Abs present in MS patients. The presence of these Abs in different MS subtypes, found at an early stage in a subset of MS patients, and increasing reactivity toward the antigenic targets with clinical disability demonstrate the diagnostic and prognostic potential of these novel biomarkers. In the future, screening for Ab reactivity toward the identified antigenic targets...
may play an important role in selecting MS patients who may benefit from specific therapies such as tolerization or B cell-directed therapies (42–45).

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Disclosures

The biomarkers identified in this study are described in European patent EP07106081.8.

References