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Peptide reactivity between multiple sclerosis (MS) CSF IgG and recombinant antibodies generated from clonally expanded plasma cells in MS CSF

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Abstract

We employed 19 recombinant antibodies (rAbs) generated from clonally expanded plasma cells, and native IgG from cerebrospinal fluid (CSF) of three multiple sclerosis (MS) patients for panning with phage displayed random peptide libraries. Specific peptide epitopes/mimotopes were identified and characterized. Importantly, peptide-antibody interactions were shared by rAbs and native IgG from the same patient. Three peptides strongly interacted with at least one other MS CSF, but not to inflammatory CNS controls. Database searches revealed several protein candidates including stress proteins, cell surface proteins, and neuronal proteins. Peptides derived from the candidate proteins were recognized by rAbs. Identification of peptide epitopes/mimotopes in MS may provide clues regarding disease-relevant antigens.

Keywords

Multiple sclerosis; Random peptide library; Epitope; Mimotope

1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS). Important features of disease are the persistence of increased numbers of B-lymphocytes and plasma cells, along with bands of oligoclonal IgG in the brain and cerebrospinal fluid (CSF). In chronic infectious diseases of the CNS, OCBs are antibody directed against the causative agent (reviewed in Gilden, 2005). Recent studies have demonstrated that recombinant antibodies (rAb) generated from clonally expanded plasma cells in the CSF of patients with subacute sclerosing panencephalitis or optic neuritis are directed against the disease relevant antigens (Burgoon et al., 2005; Owens et al., 2006; Yu et al., 2009; Bennett et al., 2009). The presence of B cell follicle-like structures in the meninges of MS patients (Aloisi and Pujol-Borrell, 2006; Magliozzi et al., 2007), and the

efficacy of B cell depletion therapy with Rituximab, an antibody directed against the B cell surface marker CD20 (Hauser et al., 2008) provide evidence for a role of B cells in the pathogenesis of MS.

By fluorescence activated cell sorting (FACS) and IgG variable region sequence analysis, our lab and others demonstrated the presence of a restricted clonal IgG population and extensive somatic mutations in MS CSF CD138⁺ plasma cells (Owens et al., 1998, 2003; Ritchie et al., 2004; Monson et al., 2005), features of an antigen-driven response. Furthermore, CD138⁺19⁺ plasma blasts constituted almost 90% of the CD138⁺ cell population, suggesting that they are responsible for the majority of the antibody production in the CSF (Winges et al., 2007). By matching the immunoglobulin transcriptomes of B cells with the corresponding immunoglobulin proteome in MS, Obermeier et al. (2008) provided direct evidence that CSF B cells are the source of oligoclonal Ig in MS patients. More recently, von Budingen et al. (2010) reported that clonally expanded plasma cells in MS CSF produce oligoclonal bands by detecting heavy chain CDR3 idiotopes with anti-idiotypic antibodies.

Several studies have identified possible targets of the antibody response in MS by panning phage displayed random peptide libraries with CSF IgG, and found that peptide antigens in MS are specific to individual antibody response (Cortese et al., 1996, 1998, 2001; Archelos et al., 1998). We have generated rAbs from clonally expanded plasma cells in the CSF of patients with MS. Herein, we report for the first time a comparative study of peptide antigenic specificity between native IgG and rAbs generated from clonally expanded plasma cells in MS CSF.

2. Materials methods

2.1. Generation of recombinant antibody

CD138⁺ plasma cell sorting and IgG heavy and light chain variable sequence amplification were performed as described (Ritchie et al., 2004). The construction and generation of all recombinant antibodies (rAbs) in this study were as reported (Owens et al., 2009). 2.2. Biopanning, phage titration and amplification

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The PhD.-12^{TM} Phage Display Peptide Library (New England BioLabs, Beverly, MA) was used for affinity selection of specific peptides. The panning procedure was essentially as described (Yu et al., 2006a) except that MS rAb/CSF IgG at a concentration of 10 µg/ml was added to wells of Reacti-Bind^{TM} Protein A-coated clear strip plates (Thermo Scientific, Rockford, IL), and the antibodies in 50 µl Tris-buffered saline (TBS) were incubated overnight at 4 °C. The phage peptide library (1.5×10¹⁰ pfu) in 100 µl of TBST (TBS-0.5% Tween 20) was added to the wells and incubated for 1 h at room temperature. Elution of bound phage was performed at either 37 °C for 10 min (Yu et al., 2009) or at room temperature for 20 min. The affinity-selected phage were titered after each of three rounds of panning. Phage titering and amplification was as described (Yu et al., 2006a).

2.3. ELISA

2.3.1. Primary 96-well ELISA—Primary single-point 96 well ELISA was carried out as reported (Yu et al., 2006b). Individual plaques (12-24 plaques panned by each antibody) from the titration of second and third pans were picked and placed into wells of U96 deepwell^{TM} plates (Agene Nunc International, Rochester, NY) containing 500 µl of a 1:10 dilution of OD 1 or 1:100 dilution of overnight culture of *E. coli* ER 2738 in LB medium. The plate was covered with breathable sealing tape (NUNC). After 4.5 h of infection at 37

°C at 250 rpm, the DeepWell plates were centrifuged at 4000 rpm for 10 min at 4 °C in a Sorvall RT 6000 refrigerated centrifuge. Phage supernatants were transferred to a new DeepWell plate and stored at 4 °C.

For primary fast screening of potential positive phage, a microtiter plate (Corning) was coated with 50 µl of antibody in 0.1 M carbonate buffer, pH 9.5 at 1 µg/ml, blocked with 3% BSA for 1 h and washed with TBST three times. Phage supernatants (50 µl) from the DeepWell plate were added directly to individual wells and incubated for 1 h with shaking at room temperature. Plates were washed with TBST 10 times for 1 min each time, followed by incubation with 50 µl of 1:500 dilution of horseradish peroxidase (HRP)/anti-M13 antibody conjugate (New England Biolabs) in 1% BSA/Tween 20 for 1 h. The plates were washed 8 times with TBST. Bound phage was detected using HRP substrate ABTS (Vector Laboratories, Burlingame, CA). After incubation with substrate for 20-30 min, absorbance at 405 nm was determined spectrophotometrically with a Microplate Reader (Bio-Rad, Hercules, CA). All phage were screened in single point. BSA (3%)-coated wells served as negative controls.

2.3.2. Phage ELISA—To confirm positive phage results from single-point 96-well ELISA, analyses were repeated with normalized amounts of phage. MS rAb/CSF IgG (50 µl, 1 µg/ml) with irrelevant antibody as negative control was coated in duplicate in wells of an ELISA plate overnight at 4 °C. Wells were blocked with 3% BSA for 1 h and 5×10^9 selected phage was added to each well. The phage detection procedure was the same as described above. ELISA was repeated at least once. Error bars represent standard deviation.

2.3.3. Phage mediated immuno-PCR (IPCR)—Phage-mediated immuno-PCR was performed as described (Yu et al., 2007). Briefly, phage bound to corresponding antibody-coated in wells of ELISA plates (in duplicate with irrelevant antibody control) were lysed in 50 µl double de-ionized water by heating the plates at 95 °C for 15 min to release single stranded phage DNA and used as template for real-time PCR. Each real-time PCR was performed in an Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA). Each PCR reaction (20 µl) consisted of 1X power SYBR® Green master mix (Applied Biosystems), 750 nM of each M13 phage primer and 4 µl of phage template. A control reaction without template was included in each run. Thermal cycler conditions included 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 45 s. A standard curve of known phage numbers was included in each PCR run for determining the number of bound phage.

2.3.4. Dose-response and competitive inhibition ELISA—For dose-response ELISA, serial 2- or 4-fold phage dilutions were added to wells coated with respective panning rAb and an irrelevant rAb as negative control. Phage detection was as described above. For inhibition ELISA, rAbs at a concentration of 10 µg/ml were pre-incubated with corresponding serial 3-fold phage dilutions in 50 µl 1% BSA-TBST and with irrelevant phage for 30 min at room temperature. The mixture was added to wells of ELISA plates containing the same phage bound to anti M13 antibody coated the night before at 4 °C. The inhibited rAb was then detected with a 1:500 dilution of anti-Flag antibody (goat anti ECS HRP conjugated, Bethyl Laboratories, Montgomery, TX) followed by ABTS color development as described above.

2.4. Immunoblots and peptide SPOT analysis

NuPAGE Bis-Tris Mini Gels (Invitrogen, Carlsbad, CA) were used for phage SDS-PAGE analysis with 1X MOPS SDS Running Buffer. Phage peptides (1×10^{11} /well) in TBS were denatured and reduced by incubating with 1X protein sample buffer containing β-

mercaptoethanol (Pierce Biotechnology, Rockford, IL) at 95 °C for 10 min. Duplicate gels were electrophoresed for 50 min under constant voltage of 200 V. The gel was then electroblotted onto a PVDF membrane (Bio-Rad) for 60 min under constant voltage of 15 V using Trans-Blot® Semi-Dry Cell (Bio-Rad). After blocking with 1X casein/TBS (Vector Laboratories) containing 0.1% Tween 20 for 1 h, the filter was incubated with corresponding rAb/ CSF (1 µg/ml) for 1 h in 1X casein/TBS buffer with 0.1% Tween 20. Peroxidase-conjugated goat anti-human IgG (H+L) (Sigma, St. Louis, MO) at a dilution of 1:5000 was used to detect rAb binding. After incubation with each antibody, membranes were washed once with TBST for 15 min, then four more times for 5 min each. The membrane was incubated with SuperSignal® West Femto Maximum Sensitivity chemiluminescent substrate (Pierce) as recommended by the manufacturer. For detection of phage pIII protein, duplicate filters were incubated with a 1:25,000 dilution of anti-M13 pIII monoclonal antibody (New England BioLabs). A 1:25,000 dilution of goat anti-mouse IgG peroxidase conjugate (Vector lab) was used as secondary antibody followed by chemiluminescent detection with Immobilon (Millipore).

For peptide SPOT analysis, cellulose-bound peptides derived from candidate proteins were generated by SPOT synthesis from Peptide Array Facility (Kinexus Bioinformatics Corporation, Canada). The peptide blot was blocked with 3% milk containing 5% sucrose, incubated with rAb MS 02-19 #25 at 1 µg/ml, and followed by HRP-conjugated anti-human IgG incubation, and detected with either SuperSignal® West Pico or SuperSignal® West Femto chemiluminescent substrate (Pierce).

2.5. DNA sequencing and database search

Single-stranded phage DNA was purified and sequenced to deduce the amino acid sequence of the peptide. Consensus peptides were identified by sequence alignment using ClustalW (<http://www.ebi.ac.uk/clustalw/>). Peptide abundance was calculated based on the number of identical sequences of the total phage clones sequenced. For identifying candidate proteins, two of the most abundant peptides panned by both rAb and CSF from each patient were used for database search in BLAST (<http://www.ncbi.nlm.nih.gov/>) using Swiss Prot protein sequences database.

3. Results

3.1. Positive phage peptides that reacted with MS antibodies were identified by panning phage displayed random peptide libraries from each of the three MS patients

MS rAbs and native CSF IgG from three MS patients (Table 1) were studied for antigen specificity by panning phage-displayed random peptide libraries (Ph.D. 12). The 19 MS rAbs were generated by co-expressing the paired IgG heavy- and light-chain variable (V) region sequences of clonally expanded CD138⁺ plasma cell in mammalian tissue culture cells (Yu et al., 2006a; Owens et al., 2009).

Each rAb/CSF IgG was coated onto wells of protein A-coated plates and the panning procedure was as described (Yu et al., 2006a). A streamlined protocol was developed to determine phage peptide specificity after affinity selection (Fig. 1). Individual phage plaques from both the second and third pan were first amplified in U96 deepwell plates and their reactivity to panning antibody was determined by single point ELISA (96-well ELISA) using crude phage culture without further purification (Yu et al., 2006b). Potential positive clones were confirmed by duplicate ELISA with IgG1 isotype control rAb, and DNA from positive phage clones were purified and sequenced. After primary screening by ELISA, positive phage peptides were identified by all three CSF IgG (four unique peptides by MS 02-19, six peptides by MS 03-7 and four by MS 04-2, ELISA data not shown). One or more

specific peptide sequences were enriched in each panning experiment. The peptide sequence of each positive phage clone panned by either rAb or CSF IgG and their relative abundance are shown in Table 2. For each rAb/CSF IgG, the abundant peptide (multiple phage clones sharing the same sequence) as well as unique peptide sequences were identified. The putative consensus sequences were also listed at the bottom of each alignment. Although there is no identical peptides shared by rAb and the CSF IgG from the same patient, sequence homologies were found in peptides panned by rAb and corresponding CSF IgG (data not shown).

Each selected phage was named by its panning antibody (rAb/CSF IgG) followed by the location of the well from which the phage was initially identified. For example, phage rAb #25-D1 indicates the phage peptide was panned by rAb #25 in well D1, and phage CSF02-19-G3 stands for the phage peptide panned by native IgG of CSF 02-19 from well G3 (Table 3).

Both ELISA and IPCR were used to confirm specificity of the peptides. For ELISA, respective rAbs/CSF IgG and isotype control rAb from the same patient or irrelevant CSF were separately coated in duplicate onto ELISA plates, and assayed for binding to peptides that were initially positive from 96-well ELISA (Fig. 2A and C). In Fig. 2A, six of nine peptides bound specifically to panning rAb #25 from patient MS 02-19, but not to IgG1 isotype control rAb cloned from the same patient. Two peptides panned by rAb #15 bound weakly to the rAb, indicating the diverse affinity of rAbs from the same MS patient. Fig. 2C shows ELISA results of peptides panned by rAbs from patients MS 03-7 and 04-2. Phage #10-2'-B11 by rAb 03-7 #10 and phage #33-3'-E4 and #33-3'-G9 by rAb 04-2 #33 had the highest binding affinity, the remaining phage peptides had weaker reactivity to panning antibodies. This again indicates that various affinities of rAbs are present from the same patients. The IPCR (Fig. 2B and D) showed similar results but higher detection sensitivities for low affinity rAbs (rAb #15 in Fig. 2B and rAbs #33, #11, and #13 in Fig. 2D), only 10×10^7 pfu of phage were added to rAb coated protein A wells compared to 10×10^9 pfu were used for ELISA. In addition, four positive peptides by CSF IgG MS02-19, six peptides by CSF IgG MS03-7, and 4 peptides by CSF IgG MS04-2 were also identified (data not shown).

Overall, a total of 17 specific phage peptides were identified by seven rAbs, and 14 peptides by CSF IgG from three MS patients (Table 2). Of the 19 MS rAbs used for panning experiments, 3/9 (33%) rAbs from patient MS 02-19 and 3/8 (37.5%) rAbs from patient MS 04-2 selected positive phage peptides. Both rAbs from patient MS03-7 selected positive phage peptides (Table 3).

3.2. Characterization of MS phage peptides

To further characterize affinity-selected peptides, dose-response and competitive inhibition assays were performed. Representative phage peptide selected by rAb from each patient (phage D1 by rAb MS 02-19, phage B11 by rAb MS 03-7 and phage C9 by rAb MS 04-2) were used for the characterization study. Each peptide was shown to bind to panning rAb but not to irrelevant rAb in a dose-dependent manner (Fig. 3). For competitive inhibition assay, phage peptide in solution phase competitively inhibited binding of panning rAb to the same peptide coated in the solid phase; the negative control irrelevant phage peptide did not inhibit binding (Fig. 4).

3.3. Both linear and conformational epitopes of MS antibodies were identified by Western blotting

Six phage peptides from three patients (one rAb- peptide and one CSF-peptide from each patient) were examined in Western blot to determine the conformational nature of their

binding. Phage were lysed and separated on a gradient of 4-12% SDS polyacrylamide gel, blotted to a PVDF membrane and probed with corresponding antibody. Three MS antibodies (rAb MS02-19 #25, CSF IgG MS 02-19 and rAb MS03-7 #10) bound to the corresponding peptides, shown as a 64 kD band that resulted from fusion of the peptide to the pIII minor coat protein of M13 phage (Fig. 5 upper panel: lanes 1-3). The other three MS antibodies (CSF IgG 03-7, rAb MS 04-2 #13 and CSF IgG MS 04-2), failed to recognize their corresponding peptides (Fig. 5 upper panel: lanes 4-6). Western blot results indicated that peptides recognized by rAbs MS02-19 #25, MS03-7 #10 and CSF IgG MS 02-19 represented linear epitopes, in contrast to peptides selected by rAb MS 04-2 #13, CSF IgG MS 03-7 and MS 04-2 which recognized conformational epitopes sensitive to denaturation. The positive control of anti-pIII antibody incubated with a duplicate filter showed the minor coat protein of 64 kD band for all six phage peptides (Fig. 5 lower panel).

3.4. Cross-reactivity of phage peptides selected by rAb and native CSF IgG from the same MS patient

A recently developed highly sensitive technique termed phage-mediated real-time immuno-PCR (IPCR) (Yu et al., 2007) was applied to determine cross reactivity of phage peptides panned by rAb and corresponding CSF IgG. A cross reactive phage can bind not only to the panning antibody (rAb/native IgG), but also to corresponding IgG/rAb from the same patients. The experiment was designed to see if a peptide panned by rAb binds to CSF IgG of the same patient and vice versa. Representative phage peptides (10^9 pfu) from two MS patients were added to wells of protein A plates coated with panning rAb/ native IgG from the same patient. Bound phage was lysed and phage DNA was examined by RT-IPCR using SYBR dye and M13 phage specific primers (Fig. 6). The data were consistent that from each patient phage peptide panned by rAb bound specifically to corresponding native CSF IgG, but not to inflammatory control CSF, while peptide selected by native CSF IgG bound only to rAb cloned from the same patient. For example, in patient 02-19, phage 02-19 #25-D1 panned by rAb #25 bound specifically to CSF 02-19 IgG, but not to IC IgG (Fig. 6A); and phage CSF 02-19-G11 panned by CSF 02-19 IgG bound specifically to rAb 02-19 #15, but not to IC IgG (Fig. 6B); in patient MS 03-7, phage 03-7 #10-B11 panned by rAb #10 bound specifically native CSF IgG, but not to control CSF (Fig. 6C), and phage CSF-A2 bound to rAb 03-7 #13 but not to control CSF (Fig. 6D). Furthermore, peptides panned by rAbs and CSF IgG from the same patient shared sequence homologies (Fig. 6E).

3.5. MS CSF and serum screening of antibody reactivity to individual peptides

To see if the rAb/CSF-selected peptides reacted with CSF from multiple MS patients, RT-IPCR screened 34 MS CSF and 26 inflammatory (IC) control CSFs with the most abundant phage peptides. The abundance of peptide was defined as the number of the identical peptides of the total peptides identified by each antibody. The most abundant peptides were those with the highest abundance. For example, in Table 2, peptide D1 (02-19 #25-D1) was the most abundant peptide panned by rAb #25 (abundance: 5/10); and peptide G9 (02-19-G9) was the most abundant one selected by CSF 02-19 (abundance: 5/9). Evaluation of 12 representative peptide (4 from each patient) bindings to 34 MS and 26 non-MS inflammatory CNS disease control CSF samples showed that three antigenic peptides strongly interacted with at least one other MS CSF but not to the IC controls (Fig. 7, Table 4). To determine whether peptide reactivity was unique to MS CSF, paired CSF and serum from the same patient were tested for peptide reactivity using highly sensitive phage-mediated IPCR. Bound phage was lysed and used as templates for (RT)-PCR. A higher number of bound phage indicates higher binding affinity. Peptides bound to MS CSF were 2.4 to 4 cycle thresholds (Ct) less than that to sera (one Ct equals to 2.2 fold difference), indicating that there were 5-10 times higher number of phage bound to CSF than sera which suggested that these peptides were specific to intrathecally-synthesized IgG (Fig. 7).

3.6. MS peptide sequences and protein database search and candidate protein conformation

Peptides with high abundance and high binding values were used to search a protein database (Swissprot) to identify corresponding protein candidates. Because of the short length of the peptide sequence, numerous candidate proteins were revealed. Major candidates included human stress proteins, membrane and cell surface proteins and neuronal proteins (sequence identities ranged 63%-100%). Peptides aligned to heat shock proteins were identified by CSF IgG from patient MS 02-19 and MS 04-2 (Table 5). We further synthesized peptides derived from four candidate proteins shared high sequence homologies with peptide rAb 02-19 #25-D1 using SPOT technology (Kinexus Bioinformatics), and probed the peptide blot with rAb #25. Two candidate peptides (plexin domain-containing protein 1 and glycogen synthase) were recognized by rAb #25 (Fig. 8A). These data demonstrated the feasibility of using MS rAb and phage libraries to identify MS protein antigens.

We further synthesized four candidate peptides derived from the database search with peptide rAb 02-19 # 25-D1 using SPOT technology, and the blot was detected with rAb 02-19 #25 followed by anti-human IgG incubation. MS rAb 02-19 #25 recognized candidate peptides derived from plexin domain-containing protein 1 and glycogen synthase (Fig. 8).

4. Discussion

Although the antigenic specificity of intrathecally produced IgG in MS is unknown, increasing evidence supports the role of humoral immunity in disease pathogenesis. We generated 19 monoclonal recombinant antibodies (rAbs) by co-expressing paired heavy- and light-chain variable sequences of single plasma cells from the CSF of three patients with MS. Several immunoassays revealed that these rAbs are not directed against three abundant myelin proteins (Owens et al., 2009). Because phage-displayed random peptide libraries contain an extremely large number of different amino acid combinations of a defined length and can readily be screened for binding to monoclonal antibodies (Scott and Smith, 1990), we analyzed antigenic peptide repertoires of 19 rAbs and corresponding native IgG from the same 3 MS patients. The 19 rAbs and 3 CSF IgGs identified 31 unique phage peptides.

The rigor of panning procedures may have generated selection of antigen peptides with higher binding affinities. For example, while all 3 CSF IgGs identified specific phage peptides, 3/9 (33%) rAbs from patient 02-19 and 3/8 (37.5%) rAbs from MS patient 04-2 selected positive clones (Tables 2 and 3). Since MS CSF contains multiple IgG species, it is not surprising that positive peptides were isolated from each CSF. Of the peptides selected by rAbs, the affinity varied considerably, indicating the heterogeneous nature of antigen binding properties (Figs. 2, 3 and 4). MS peptides with high binding affinity are likely to be useful to investigate the specificity of oligoclonal IgG and their role in the etiology and pathogenesis of MS.

B cell epitopes are linear or conformational. Our immunoblotting analyses (Fig. 5) suggest that both linear and conformational epitopes of MS antibodies were identified. For example, rAb (02-19 #25) and CSF IgG from MS patient 02-19 selected linear epitopes (Fig. 5, lanes 1 and 2). Identification of linear epitopes provides a useful tool to investigate the corresponding protein antigens against which MS antibodies are directed. The other two MS CSFs did not identify linear epitopes, possibly because they were conformational. Nevertheless, conformation epitopes (or mimotopes) cannot be overlooked because they too may be valuable tools for identifying disease markers and for predicting disease progression.

When the rAbs and the CSF IgG from the same patients were screened on the phage libraries by ELISA, the peptide hits panned by the rAbs were not the same as the peptide hits panned by native CSF IgG. This indicates that the high affinity antibodies contained in the CSF IgG were not recognizing the same peptides as the rAbs generated from the same patient's CSF plasma cells. However, phage peptides selected by rAbs reacted to native IgG of the same patient determined by the highly sensitive phage-mediated IPCR (Fig. 6). Conversely, peptides selected by native CSF IgG from MS patients bound to rAbs from the same patient, but not to non-MS inflammatory control CSF IgG. The peptide cross-reactivity obtained by phage IPCR indicate that IgG cross-reactive to peptides with lower affinity. Obermeier et al. (2008) showed that MS CSF IgG proteomes match with the corresponding B cell Ig transcriptomes, indicating that CSF B cells are a source of the immunoglobulin proteome of subject-specific oligoclonal IgG. Cross-reactivity of phage peptides between rAbs and native IgG further supports the notion that rAbs from expanded plasma clones in CSF share antigens that react with the native IgG. Because a single antigen contains multiple epitopes, it is possible that the peptides identified by rAbs and native CSF belong to the same protein antigen. In addition, the results of phage-mediated IPCR of phage to paired CSF and serum suggested that the peptides were specific to intrathecally-synthesized IgG (Fig. 7).

Several groups have identified possible targets of the antibody response in MS by panning phage displayed random peptide libraries with CSF IgG, and found that peptide antigens in MS are specific to individual antibody response (Cortese et al., 1996, 1998, 2001; Archelos et al., 1998). Although our peptides had no identical or overlap identities with theirs, the aligned protein candidates shared the same autoantigen categories such as membrane and cell surface proteins, neuronal proteins, and human stress proteins. Although other groups have demonstrated myelin-reactivity of CSF or rAbs generated from MS patients (Fraussen et al., 2009; McLaughlin and Wucherpfennig, 2008; Villar et al., 2005; Zhang et al., 2005a, 2005b; Reindl et al., 1999; Lampasona et al., 2004; Zhou et al., 2006; Lalive et al., 2006; Berger et al., 2003; Kuhle et al., 2007; O'Connor et al., 2003, 2007; Lambracht-Washington et al., 2007; von Büdingen et al., 2008; Genain et al., 1999), our peptides did not align to any myelin proteins. This may be due to the small sample size studied, and most of the peptides represented conformational epitopes or mimotopes of myelin proteins.

Furthermore, several peptides reacted with at least one CSF from a different MS patient, but not with serum from that patient or with CSF from control patients with non-MS CNS inflammatory diseases, revealing that intrathecally synthesized IgG from different MS patients recognizes shared epitopes/mimotopes. Alignments of high-affinity peptides to the SwissProt protein database revealed a plethora of candidate proteins, including human stress proteins, membrane and cell surface proteins and neuronal proteins (Table 5). None of the peptides aligned to myelin protein antigens, consistent with our previous finding (Owens et al., 2009). In addition, MS rAbs bound to candidate protein peptides derived database search (Fig. 8). Further study is needed to investigate how these candidate proteins may influence pathogenesis of the disease.

Compared to our previous report (Yu et al., 2006a, 2006b), significant progress was made in the current study. 1) In the 2006 paper, no high affinity peptides were identified by rAbs of patient MS 02-19, and no further characterization was made. We have optimized the peptide screening protocol, and high affinity peptides (02-19 #25-D1 by rAb MS02-19) were identified and characterized in the current manuscript. 2) major findings in the current report include that high affinity peptides were identified and characterized by 19 rAbs and native CSF IgG from each of 3 MS patients, peptide reactivity of the rAbs and CSF IgG pool are similar, and candidate protein peptides were recognized by MS rAbs. Although our peptides had no identical or overlap identities with findings of others (Cortese et al., 1996, 1998,

2001; Archelos et al. 1998), the aligned protein candidates shared the same autoantigen categories such as membrane and cell surface proteins, neuronal proteins, and human stress proteins. Importantly, we found that several peptides reacted with at least one CSF from a different MS patient, but not with serum from that patient or with CSF from control patients with non-MS CNS inflammatory diseases, revealing that intrathecally synthesized IgG from different MS patients recognizes shared epitopes/mimotopes.

Monoclonal rAbs derived from clonally expanded CD138⁺ plasma cells in CSF provide a unique tool for antigen identification in MS for a number of reasons. First, MS rAbs replicate the paired IgG heavy and light chains from antibody-secreting plasma blasts (Winges et al., 2007). Second, antigen-binding sequences of MS rAbs can be readily characterized. Finally, unlimited amounts of MS rAbs can be produced. Overall, MS rAbs have considerable potential to identify disease-relevant antigens.

In summary, we have demonstrated that phage-displayed random peptide libraries can successfully identify epitopes or mimotopes of MS antibodies, and that these peptides share antigenic interactions between rAbs and native IgG within each patient. MS peptides with high affinity bindings can readily be employed to investigate the specificity of the oligoclonal IgG in MS brain and CSF to study the etiology and pathogenesis of disease.

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Fig. 1.

Streamlined protocol to determine specificity of phage peptides. Individual phage plaque from 2nd and 3rd pans are amplified in U96 deepwell plates, and initially screened for positivity against panning antibody using ELISA (96 well single point ELISA). Potential positive clones were confirmed by duplicate ELISA/IPCR with isotype control rAb as negative control, followed by phage DNA purification and sequence analysis.

Fig. 2. ELISA/PCR confirmed positive phage peptides. For ELISA (A and C), phage peptides were amplified ($\sim 1 \times 10^9$ pfu) and added to wells of ELISA plates pre-coated with panning

rAb or IgG1 isotype control rAb from the same patient. Bound phage was detected by HRP-conjugated anti-M13 antibody followed by addition of HRP substrate ABTS for color development. Positive phage were those that had at least twice the OD value of the negative control. For IPCR (B and D), crude phage ($\sim 1 \times 10^7$ pfu) from culture of U96 Deepwell plates were added directly to Protein A wells coated with panning rAb or BSA, and the bound phage was quantified by real-time PCR with M13 primers. Positive phages were those that had at least 1.4 CT less than that of the BSA control. The ELISA/IPCR were performed in duplicate and repeated at least once. Error bars represent standard deviation. Phage panned by two rAbs (#15 and #25) from patient MS02-19 are shown in Fig. A and C; phage by rAbs from patients MS03-7 and MS 04-2 are shown in Fig. B and D.

Fig. 3.

Phage peptide binds to MS rAbs in a dose-responsive manner. MS rAbs (1 µg/ml) from each of the 3 MS patient were coated onto wells of ELISA plates. Serial dilutions of corresponding phage peptides were added to each well. Bound phage was detected by HRP-conjugated anti M13 antibody followed by ABTS color detection. Irrelevant rAb from the same patient served as isotype control.

Fig. 4.

Competitive inhibition ELISA confirms binding specificity of selected phage peptides. Phage peptides ($\sim 1 \times 10^{10}$) were added to ELISA wells coated with anti M13 antibody. MS rAb ($10 \mu\text{g/ml}$) pre-incubated with serial 3-fold dilutions of phage were added to each well. Bound rAb was detected by HRP-conjugated anti-Flag antibody. Binding of phage peptide to MS rAb in solid phase was inhibited by the same phage in solution, but not by an irrelevant phage peptide (4A: phage #25-D1 to rAb MS 02-19 #25; 4B: phage #10-B11 to rAb MS 03-7 #10; 4 C: phage #13-C9 to rAb MS 04-2 #13).

Fig. 5.

Western blot demonstrates that some MS peptide epitopes are linear. All MS rAbs/CSF which selected positive peptides were used to evaluate bindings to linear peptides in Western blots. Shown are six phage peptides panned with both rAbs and CSF IgG from each of the three patients were separated on a gradient of 4-12% SDS polyacrylamide gel, blotted to a PVDF membrane and incubated with corresponding rAb and CSF IgG. Phage peptide D1 (lane 1), H3 (lane 2) and B11 (lane 3) bound specifically to panning antibodies rAb 02-19 #25, CSF02-19 IgG and rAb 03-7 #10, respectively (top panel, lanes 1, 2, 3), indicating that they are linear epitopes. No bands were seen with phage A2 (lane 4), C9 (lane 5) and E12 (lane 6) possibly because they may represent conformational epitopes. Anti-pIII antibody incubation with a duplicate membrane (lower panel) showed phage loading bands with all six phage peptides.

Fig. 6.

Phage peptides selected by rAb and corresponding CSF IgG cross react. CSF IgG (5 µg/ml) or representative MS rAb (1 µg/ml) from patients MS 02-19 (A and B) and MS 03-7 (C and D), as well as inflammatory control CSF IgG were coated onto wells of protein A plates. Phage (10^9) panned by rAbs (A and C) were added to wells containing corresponding CSF IgG, and phage panned by CSF IgG (B and D) were added to wells containing representative rAb of the same patients. Bound phage was lysed by heating the ELISA plate at 95 °C for 15 min, and the phage number was determined by phage-mediated Immuno-PCR. All IPCR were performed in duplicate and repeated at least once. Fig. 6E shows that peptides panned by rAbs and CSF shared sequence homologies. Top panel: MS 02-19; Lower panel: MS 03-7. The identical residues are in bold and marked with symbol “*”. Symbols “.” and “.” indicate identical, conserved, and semi-conserved residues respectively.

Fig. 7.

Phage peptides are specific for intrathecally-synthesized IgG in MS. MS CSF and paired serum IgG (5 µg/ml) were coated onto wells of protein A plates. Phage (10^9) were added to the IgG coated wells, and bound phage was lysed and used as templates for real time PCR. A greater number of bound phage indicates a higher binding affinity. Peptides bound to MS CSF were 2.4 to 4 cycle thresholds (Ct) less than that to sera (one Ct equals to 2.2 fold difference), indicating that there were 5-10 times more phage bound to CSF than to sera. Pre-immune human IgG was used as a negative control. All immuno-PCRs were performed in duplicate and repeated at least once.

Fig. 8.

MS rAbs bind to candidate protein peptides identified by database search. A. Four candidate peptides identified from a database search with phage peptide rAb 02-19 #25-D1 were synthesized by SPOT synthesis technology, and probed with rAb 02-19 #25 followed by anti-human IgG incubation and chemiluminescent detection. Peptides derived from candidate proteins plexin domain-containing protein 1 and glycogen synthase were recognized by rAb 02-19 #25. Sequence alignments are shown on the right. A star (*) indicates identical amino acid residues and a semicolon (;) indicates conserved amino acid residues. B. lists candidate peptide sequences testing for binding to rAb #25.

Table 1

Clinical features of MS patients.

Patient diagnosis	MS02-19 (primary progressive)	MS03-7 (relapsing-remitting)	MS04-2 (relapsing-progressive)
White matter lesions	+	+	+
Number of WBC ^a	10	10	0
% CSF IgG ^b	15	32	21
Oligoclonal bands	+	+	+
Number of clonal populations ^c	8	11	8
Number of rAbs generated ^d	9	2	8

^a White blood cells per cubic millimeter.^b The percentage of total protein in CSF that corresponds to IgG.^c Determined by FACS-single cell PCR (Ritchie et al., 2004).^d rAbs generated from clonal expanded plasma cells (Yu et al., 2006a).

Table 2

Positive phage peptides selected by MS rAbs and CSF IgG.^a

Patient	Antibody	Phage	Peptide	Abundance
02-19	rAb #25	D3	S C N Y H H T Y L T C W	1/10
		D1	N C S W K G H V L Q C S	5/10
		D2	N C S W K G H V L Q C S	
		D9	N C S W K G H V L Q C S	
		D10	N C S W K G H V L Q C S	
	rAb #15	D7	N C A F W L G R L Y C R	1/10
		D3	S C Q L V R T I W S C S	1/10
		D4	G T W I C D F K S C S W	1/10
		Consensus	C S W K G H V L S C S	
		A10	N N P A P V Y T H R V L	1/3
	CSF IgG	A9	F H Q P T Y D Y T V S R	1/3
		G9	N N L T Q S K F L R L Q	5/9
		G11	N N L T Q S K F L R L Q	
		G11'	N N L T Q S K F L R L Q	
		H6'	N N L T Q S K F L R L Q	
		G8'	N N L T Q S K F L R L Q	
		H2	N T L T P H K L Q M L	1/9
		H3	S T L S E S K V N R L	2/9
		H3'	S T L S E S K V N R L	
		G3	N A L T E S K Y V K L L	1/9
		Consensus	N N L T S Q R S K F L R L	
03-7	rAb#10	B10'	D P G Q L R L W R L A S	4/6
		B11	D P G Q L R L W R L A S	
		B3	D P G Q L R L W R L A S	
		B7	D P G Q L R L W R L A S	
		B10	Y P S W L H A Y R L A	
		B9'	Q P T W L Q V I	1/6
		Consensus	N P G Q L R L W R L A	1/6
	rAb#13	E8	H T Q P Y A Y E A R D H	3/7
		E4	H Y N P W E Q E P M M Y	1/7
		Consensus	H T Q P Y A Y E A R D H	
	CSF IgG	B12	S L G S K M D I S R P W	2/13
		A2	S L G S K M D I S R P W	
		B6	T I M D I S R T W T K V	1/13
		A7	T A Q D I S R P W W F P	2/13
		A3	H I D V S R P W R V T G	3/13
		B1	H I D V S R P W R V T G	
		B3	H I D V S R P W R V T G	
		A11	H I D V S R P W R V T G	
		A5	Q H N V S R P W V L F T	1/13
		Consensus	D/N I/V S R P W	
	rAb #13	D3	V P Q K Y W W L S D H T	5/8
		C2	V P Q K Y W W L S D H T	
		C4	V P Q K Y W W L S D H T	
		C9	V P Q K Y W W L S D H T	
		C12	V P Q K Y W W L S D H T	
		D2	V V P K Y W W S A E L P	1/8
		Consensus	V P Q K Y W W L S D/E	
	rAb #33	G1	S V S V G M K P S P R P	8/8
		G2	S V S V G M K P S P R P	
		G3	S V S V G M K P S P R P	
		G4	S V S V G M K P S P R P	
		G7	S V S V G M K P S P R P	
		G9	S V S V G M K P S P R P	
		G11	S V S V G M K P S P R P	
		G12	S V S V G M K P S P R P	
		Consensus	S V S V G M K P S P R P	
04-2	rAb #92	B3	T Q G L K Y W Y P V W S	2/2
		B12	T Q G L K Y W Y P V W S	
		Consensus	T Q G L K Y W Y P V W S	
	CSF IgG	E8	N P V E H W L A V L P T	1/6
		E8'	H W R H W L A D T A F P	1/6
		Consensus1	H W L A D T A L P	
	CSF IgG	E7'	F S K T E P L S P S W F	3/6
		E7'	F S K T E P L S P S W F	
		E12'	F S K T E P L S P S W F	
		F4	N N L T Q S K F L R L Q	1/6
		Consensus	N N L T Q S K F L R L Q	

^aDNA of positive phage from second and third rounds of panning were purified and sequenced. Peptides selected by each rAb/CSF IgG were aligned using Clutal W multiple alignment program. The identical sequences were boxed. “&”,”,” indicate identical, conserved, and semi-conserved residues respectively. The putative consensus sequences are listed at the bottom of each alignment.

Table 3

MS recombinant antibodies and CSF IgG that identified positive phage peptides.

Patient	MS antibodies that identified positive peptides	# of peptides identified	Peptide nomenclature ^a
Patient #1 (MS02-19)	rAb 02-19 #25	10	Peptide rAb 02-19 #25-Xn
	rAb 02-19 #15	3	Peptide rAb 02-19 #15-Xn
	rAb 02-19 #11	2	Peptide rAb 02-19 #11-Xn
	CSF 02-19 IgG	9	Peptide CSF 02-19-Xn
Patient #2 (MS03-7)	rAb 03-7 #10	6	Peptide rAb 03-7 #10-Xn
	rAb 03-7 #13	7	Peptide rAb 03-7 #10-Xn
	CSF 03-7 IgG	13	Peptide CSF 03-7-Xn
Patient #3 (MS04-2)	rAb 04-2 #11	1	Peptide rAb 04-2 #11-Xn
	rAb 04-2 #13	8	Peptide rAb 04-2 #13-Xn
	rAb 04-2 #33	8	Peptide rAb 04-2 #33-Xn
	CSF 04-2 IgG	6	Peptide CSF 04-2-Xn

^aEach phage peptide was named by its panning antibody (rAb/CSF) followed by the location of the well from which the phage was initially identified. For example, peptide rAb 02-19 #25-D1 indicates that the phage peptide was panned by rAb #25 in well D1, and peptide CSF02-19-G3 stands for the phage panned by CSF IgG of patient 02-19 from well G3. “X” indicates any letter from A-H, and “n” indicates any number from 1 to 12.

Table 4

IPCR screening of MS and non-MS inflammatory CNS control (IC) CSFs with individual phage peptides.^a

Phage peptides	MS CSF ^b	IC CSF ^c
CSF 02-19-G3	2/34 (+++)	0/26
CSF 02-19-G11	2/34 (+++)	0/26
rAb 03-7 #13-E4	2/34 (+)	1/26 (+)
CSF 03-7-A2	3/34 (++)	0/26
CSF 04-2-E12	2/34 (+)	1/26 (+)

^a Individual phage peptide were screened for reactivity to both MS and control CSF using phage mediated Immuno-PCR with preimmune human IgG as negative control.

^b Number of positive CSF of the total CSFs tested. CSF was considered positive when the value of cycle threshold (Ct) in real time PCR was at least 1 Ct smaller than the negative control. “+++” indicates strong binding.

^c Two of six MS phage peptides bound to the inflammatory control CSF from a patient with limbic encephalitis.

Table 5 Peptide alignment to protein database.^a

Patient	Peptide	Homology to known proteins	Identities	Accession number ^b
MS02-19	rAb#25-D1	-Plexin domain containing 1, isoform CRA_b	10/13 (76%)	gb EAW60552.1
		-Human papillomavirus type 6b E6 protein	7/11 (63%)	sp P06462
	CSF-H3	-Heat shock 70 kDa protein 1	9/11 (81%)	sp Q9ZEJ6
		-Zinc finger protein rst2	7/7 (100%)	sp P78871
MS03-7	rAb#10-B11	-Ankyrin repeat and death domain containing 1A	9/11 (81%)	sp Q495B1.1
		-Voltage-gated hydrogen channel 1	6/7 (85%)	sp Q9 .1
	rAb#13-E1	-Annexin VIII	5/8 (62%)	sp O35640.1
		-Zinc finger and BTB domain-containing protein 7 C	5/6 (83%)	sp A1YPR0.1
	CSF-A11	-Myeloma over expressed gene protein	7/10 (70%)	sp Q96EZ4
		-Neuron-derived orphan receptor 1	6/7 (85%)	sp Q92570.3
MS04-2	rAb#13-C2	-Collagen, type III, alpha 1	5/7 (71%)	sp P02461
		-Chaperone protein torD	5/5 (100%)	sp Q5E4I0
	CSF-E12	-Heat shock factor protein	7/8 (87%)	sp P41154
		-Natural-killer cells cyclophilin-related protein	7/8 (87%)	sp P30415

^a Representative peptides selected by both rAb and CSF IgG from each patient were aligned to Swissprot protein sequence database using BLAST (<http://www.ncbi.nlm.nih.gov/>). The peptides were those with the highest ELISA value or frequency. Two candidate proteins with significant identities to MS peptides were listed from each peptide alignment.

^b gb: genebank. sp: Swissprot.