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Identification of peptides specific for cerebrospinal fluid antibodies in multiple sclerosis by using phage libraries

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ABSTRACT **The study of the origin and pathogenetic relevance of the oligoclonal antibodies present in the cerebrospinal fluid (CSF) of multiple sclerosis (MS) patients has been hampered by a lack of specific ligands. We recently reported a general strategy, based on phage-displayed random peptide libraries, to identify ligands for disease-specific antibodies even in the absence of any information on the nature of the pathologic antigen. With this procedure, we identified several peptides specifically recognized by antibodies present in the CSF of MS patients. Using these peptides as reagents, we demonstrated that they mimic different natural epitopes and react with antibodies enriched in the CSF of MS patients. Antibodies recognizing the selected peptides are commonly found with equal frequency in the sera of MS patients and of normal individuals. In contrast, the repertoire of CSF antibodies appears to be individual-specific and is probably the result of a nonspecific immunodysregulation rather than a stereotyped response to a single antigen/agent.**

Diagnosis of multiple sclerosis (MS) is often supported by the presence of oligoclonal antibodies in the patients' cerebrospinal fluid (CSF). This is obtained by isoelectric focusing (IEF) of equal amounts of total IgG from crude CSF and serum samples of the same patient, showing the presence of several discrete bands [named oligoclonal bands (OB)] in the CSF but not in the serum. Such selective increases of CSF IgG with restricted heterogeneity are found in about 95% of MS patients (1). Kinetic studies of IgG turnover within the central nervous system (CNS), the CSF–IgG index, and comparative CSF–serum κ/λ chain ratio studies all argue strongly in favor of intrathecal synthesis of the oligoclonal IgG in MS (2–6). The IgG patterns differ from one patient to another but remain fairly constant in each individual during the course of the disease (7–10) and do not appear to be affected by treatment with immunomodulatory drugs (11). These data seem to imply a specific immune response, developed within the CNS, against persistent epitopes.

OB antibodies can also be found in other inflammatory neurological diseases such as tuberculous meningitis, neurosyphilis, progressive rubella panencephalitis, subacute sclerosing panencephalitis, and others (8, 12–16). In these pathologies, the majority of the oligoclonal IgG has been found to recognize the etiologic agent (17), whereas for MS there is no general consensus on the nature of the antigens that react with the oligoclonal antibodies present in the CSF (18–21). The failure to identify the natural antigens binding to the OB antibodies has brought the study of their origin and pathological relevance to a standstill.

Over the last few years, random peptide libraries (RPLs) displayed on phage have been used as a source of ligands to antibodies and receptors. These ligands are not necessarily identical or even similar to the natural ones, but mimic their binding properties. RPLs have been extensively used to select peptides mimicking linear epitopes or folded protein domains, and even nonproteinaceous molecules (22–24). Since the binding properties of the selected peptides to antibodies are usually determined when they are displayed on the phage particle, we have introduced the term phagotope to refer to this special type of phage displayed epitope.

Using a pool of sera from rheumatoid arthritis (RA) patients, Dybwad *et al.* (25) have been able to identify phagotopes displaying a higher frequency of reactivity with antibodies present in RA sera than in normal control sera. Recently, we have developed a novel and general strategy to identify phagotopes that bind to disease-specific antibodies present in the serum of patients that avails itself only of sera from patients and control individuals and does not rely on any information regarding the natural antigens (26). In the present work, we have applied this strategy to selecting phagotopes that are recognized by IgG present in the CSF of MS patients.

MATERIALS AND METHODS

CSF and Sera. CSF and sera from patients (37 females and 18 males; ages between 20 and 53 years, with a mean age of 35 years) with diagnosis of definite MS (27) and shown to have OBs were classified as CSF and S, respectively. Sera from healthy volunteers were classified as N. CSF used for the affinity selection and the immunoscreening had IgG concentrations of 50 μg/ml (CSF1) and 32 μg/ml (CSF2).

Affinity Selection and Immunological Screening. Magnetic beads (Dynabeads M-450, Tosyl-activated, Dynal, Oslo) were coated with anti-human Fc-specific antibody (Immunopure goat anti-human IgG Fc-specific, Pierce) at a concentration of 75 μg/ml in borate buffer for 12 h at 4°C on a rotating wheel. After washing with PBS/0.1% BSA, beads were blocked in the same buffer overnight. The beads were then incubated with CSF (5 μg of IgG) in 2× PBS/0.2% BSA/0.2% Tween 20 for 12 h on rotating wheel at 4°C. Beads were washed and then preincubated with an excess of UV-killed M13K07 phage (2×10^{13} phage particles) in PBS/0.05% Tween 20 containing BSA (1 mg/ml) for 4 h at 4°C. Approximately 2×10^{11} library phage were then added to the above preincubation mixture and incubated for 12 h at 4°C. Unbound phage were removed and the beads were washed extensively with PBS at 4°C. Bound phage were eluted by incubating the beads with 500 μl of elution buffer [0.1 M HCl, adjusted to pH 2.2 with glycine/BSA (1 mg/ml)] for 30 min with gentle agitation at room temperature. The eluate was transferred to a polypropylene tube and neutralized with 50 μl of 2 M Tris·HCl (pH 9.0). Unbound and eluted phage were titrated by infection of

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Abbreviations: CSF, cerebrospinal fluid; MS, multiple sclerosis; IEF, isoelectric focusing; OB, oligoclonal band; CNS, central nervous system; RPL, random peptide library.

XL1-blue bacteria. The percentage of clones containing a productive insert was determined by plating infected bacteria on X-Gal/IPTG indicator plates (28).

Enriched phage pools were then immunoscreened with the CSF used during affinity selection as described (26). About 10^5 phage were immunoscreened using 1:100 dilutions of CSF. Rescreening of the positive phage was performed as follows. Portions of agar corresponding to positive plaques were aspirated and collected into a Luria Bertani medium (LB)-containing sterile tube. Bacteria were killed by heat treatment at 70°C for 20 min and removed (with the agar) by centrifugation. Phage-containing supernatant was used to infect XL1-blue cells. Thirty minutes after infection, bacteria were superinfected with M13KO7 phage at a multiplicity of infection of 30 and further incubated for 30 min at 37°C. The infection mixture was then plated onto LB plates supplemented with 1% glucose (glu), ampicillin (Amp; 100 mg/ml), and kanamicin (Kan; 20 mg/ml). After overnight incubation Amp-resistant/Kan-resistant (Amp^r/Kan^r) colonies were transferred to a new Amp/Kan/glu plate according to an ordered grid (master plate). After growth on this master plate, phage-producing colonies were replicated onto a lawn of XL1-blue cells with a custom-made stainless steel multipin matrix. Nitrocellulose filters were layered onto these plates and immunoscreened.

ELISA Assays. ELISA assays using phage supernatants and human CSF and sera were performed as described (29). ELISA wells were coated with mAb anti-pIII at a concentration of 1 μg/ml. Phage supernatant was used at a 1:2 dilution in blocking buffer (5% nonfat dry milk/0.05% Tween 20 in PBS). CSF and sera were diluted 1:100 (unless otherwise indicated) in blocking buffer. Goat anti-human IgG (Fc-specific) alkaline phosphatase-conjugated immunoglobulins (Sigma), diluted 1:5000 were used as secondary antibodies.

For competition assays, 20 μl of competing phage supernatant prepared using the f1 11.1 mutant (29) was preincubated for 2 h at room temperature with the CSF.

For the OB antibody ELISA, total IgG concentrations in CSF and serum were determined by ELISA as follows. Wells were coated with anti-human IgG Fc-specific antibodies (Pierce) at 1:5000 dilutions. After washing and blocking serial dilutions of the CSF and serum samples were incubated for 2 h at room temperature. Plates were washed and human IgG were detected by incubation with anti-human IgG Fab-specific alkaline phosphatase-conjugated immunoglobulins (Sigma) diluted 1:2000. Purified human IgG were used as a standard. Serum at 3.1, 12.5, and 50 ng and CSF IgG were then used in the OB antibody ELISA.

RESULTS

Selection of Phagotopes Binding to CSF Antibodies of MS Patients. CSF samples from two MS patients (CSF1 and CSF2), with characteristic OB patterns upon IEF analysis, were used as a source of antibodies for the selection of phagotopes from two RPLs that display random nonamers as a fusion to the major coat protein pVIII, but in one case the foreign peptides are in a constrained conformation due to flanking cysteines forming disulfide bridges (pVIII9aa-cys, ref. 30).

Affinity selection and subsequent immunoscreening of the enriched phage pools led to the identification of several hundred phagotopes that were specifically recognized by IgG in CSF1 and a smaller number of phagotopes positive to CSF2. Thirty-two phage taken from those that displayed a strong reactivity with the selector CSF were studied further.

Reactivity of the selected phage with CSF antibodies was first confirmed by the quantitative ELISA assay (29). All phagotopes showed a positive signal with either CSF1 or CSF2 IgG ranging from 4 to 10 times the background values observed with wild-type phage (Fig. 1 and data not shown).

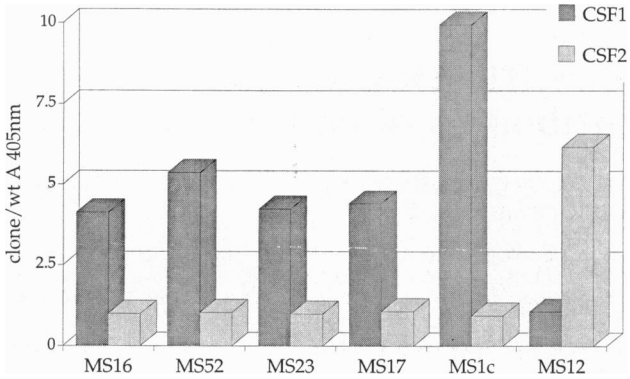

FIG. 1. Reactivity of selected phagotopes with IgG present in the CSF of two MS patients (CSF1 and CSF2). Binding of the selected phagotopes to antibodies present in MS CSF was detected by ELISA on immobilized phage. For each CSF, IgG recognition of the tested phagotope and wild-type phage was measured. Average values (A_{405}) from two experiments have been determined. Results are expressed as the ratio between the average value of the tested clone and that of wild-type phage (clone/wt A_{405}). Darkly and lightly shaded histograms represent CSF1 and CSF2 IgG reactivities, respectively. Tested phage are indicated at the bottom.

The inserts of the selected clones were sequenced, and their deduced amino acid sequences are reported in Fig. 2. The phagotopes were grouped into three classes on the basis of their amino acid homology. The majority of the CSF1-positive clones showed related sequences with a highly conserved KPPNP motif (class I: MS16, MS52, MS23, and MS17). The remaining phagotopes recognized by CSF1 IgG displayed two distinct but highly homologous sequences (class II: MS1c and MS87). Although recognized by antibodies present in the same CSF, the two classes of phagotopes have quite different amino acid sequences, suggesting they mimic distinct epitopes. This is supported by the results of cross-inhibition assays between the different clones using CSF1 in ELISA. As shown in Fig. 3, both MS16 and MS17 (class I) mutually abolished recognition by CSF1 antibodies when challenged against each other. By contrast, no inhibition was observed when MS1c (class II) was used to block recognition of MS16 and MS17 by CSF1 antibodies or in similar reactions with MS1c as target and the other two phage as competitors (Fig. 3). Consistent results were obtained using the other members of the two classes of phagotopes (data not shown). These data provided direct evidence that phagotopes belonging to class I and class II are recognized by antibodies of different specificity.

Three CSF2-positive clones were identified, corresponding to two distinct but homologous phagotopes whose sequences

		aa sequence	n. isolates	selector
class I	MS16	SRP**KPPNP**S	15	CSF1
	MS52	KTK**KPPNP**S	4	CSF1
	MS23	RL**KPPNP**TE	2	CSF1
	MS17	R**KPPNP**PPP	1	CSF1
class II	MS1c	**KRDSISPYS**	6	CSF1
	MS87	**RRDTISPYS**	1	CSF1
class III	MS42	KP**KT**NQIRP	2	CSF2
	MS12	**KKT**GNITPK	1	CSF2

FIG. 2. Amino acid sequences and classification of the selected phagotopes. Deduced amino acid sequence of the inserts of phagotopes recognized by IgG present in the CSF1 and CSF2 patients are displayed. The number of isolates for each clone is reported on the right of each sequence. Clone names are indicated on the left of their relative amino acid sequence. Phagotopes have been grouped on the basis of their sequence homology. Letters in boldface type represent conserved residues. The CSF used for selection is indicated on the right.

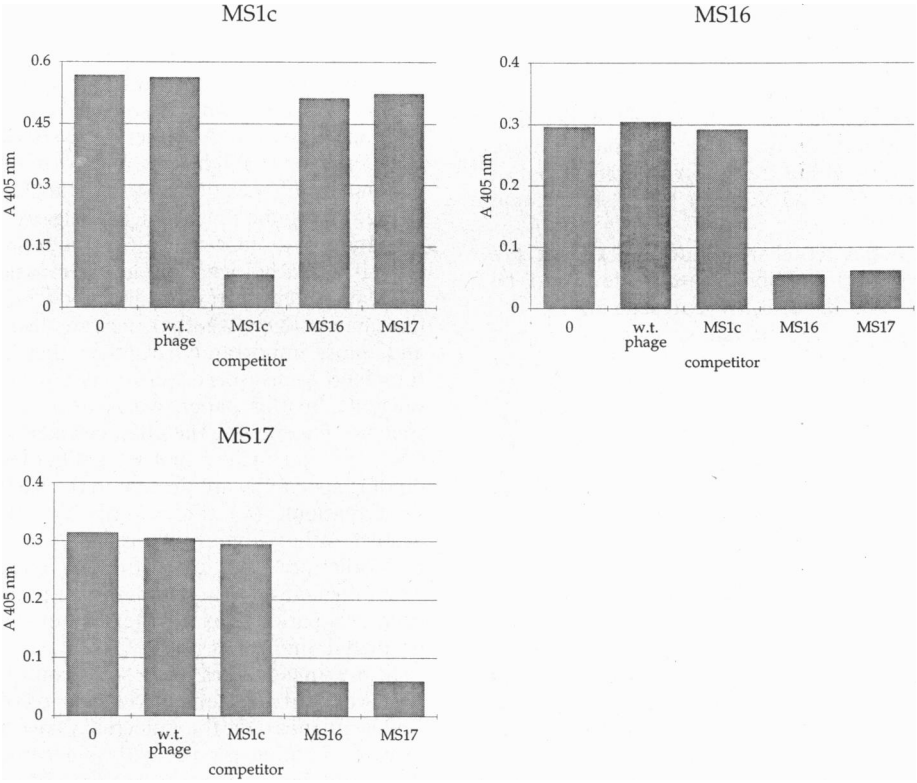

FIG. 3. CSF1-selected phagotopes mimic two epitopes. Inhibition of phagotope recognition by antibodies present in the CSF1 measured in ELISA. Immobilized phagotopes are indicated at the top of each panel. Competitor phage are indicated at the bottom. Average values from two experiments are reported.

are totally unrelated to those of the CSF1-selected phagotopes (class III in Fig. 2). As these phagotopes did not react with CSF1, it is likely that they mimic a third epitope.

Among the identified phagotopes only one (MS1c) was selected from the disulfide-constrained library. However, the efficiency of selection of this phagotope was significantly higher than that of the unconstrained sequence-related phage MS87. Therefore, it would appear that, at least for some antibodies, a more rigid conformation might favor binding of the target peptide.

The Selected Phagotopes React Both with the Serum and the CSF of the Same Patient. The absence of reagents able to detect individual CSF antibody species has up to now hampered the possibility of ascertaining whether antibodies with the same specificity are also present in the patients' sera. We have used phagotopes from each class to address this issue. Serum samples obtained from the same MS patients whose CSF recognized clones MS1c, MS12, and MS17 were tested for reactivity with the selected phagotopes by ELISA. The results of this experiment indicated that antibodies recognizing the phagotopes are also present in the patients' sera (Fig. 4).

The Phagotopes Selected with CSF1 and CSF2 Very Rarely React with CSF of Other MS Patients. Whether CSF antibodies from different MS patients display the same binding specificity is still an open issue. To answer this question, we tested the reactivity of the selected phagotopes with CSF samples from 53 additional MS patients and from 12 patients affected by neurological diseases different from MS. Clones MS16, MS17, MS1c, and MS12 were chosen to represent the three peptide classes for this analysis. Results indicated that the reactivity was essentially idiosyncratic: MS1c did not react with any of the tested samples, whereas MS16 and MS17 reacted only with one other CSF sample (CSF38). Similarly, MS12 was also recognized by antibodies present in CSF64 (data not shown). Thus, CSF IgG specific for the three families of phagotopes are very rare in the MS population, suggesting that the repertoire of CSF antibodies in MS patients is individual-specific. These results also indicate that most likely the peptides encoded by the selected phage do not mimic antigens essential to the disease state of MS patients at the time of our analysis.

The Selected Phagotopes React Frequently with the Serum of MS Patients and Normal Individuals. The results described above prompted us to make a more detailed study of the anti-phagotope reactivity of MS patients' sera. To this end, sera from all patients previously investigated for anti-phagotope reactivity in the CSF were subjected to the same kind of analysis. In contrast to the low frequency of reactivity

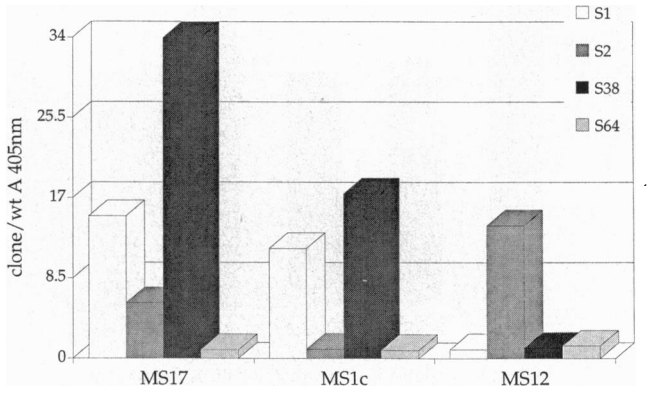

FIG. 4. Reactivity of selected phagotopes with MS patients' sera. Binding of the selected phagotopes to antibodies present in the sera of MS patients (S) was detected by ELISA on immobilized phage (indicated at the bottom). For each serum, antibody recognition of the tested clone and wild-type phage was measured. Average values (A_{405}) from two experiments have been determined. Results are expressed as the ratio between the average value of the tested phagotope and of wild-type phage (clone/wt A_{405}).

with CSF, the selected phagotopes were recognized by a large number of sera. In fact, about 10% of the tested samples reacted with MS1c and MS12 and MS17 was recognized in 58% of the samples (data not shown).

We then tested a large panel of sera from normal individuals with MS17, MS1c, and MS12. All three phagotopes were recognized by normal sera with a frequency similar to that of MS patients' sera (50%, 5%, and 25% for MS17, MS1c, and MS12, respectively).

The Selected Phagotopes React with Antibodies That Are Enriched in the CSF. OB antibodies are believed to be produced intrathecally and most likely represent highly concentrated and quantitatively predominant species with respect to the rest of the IgG present in the CSF, hence, their appearance as distinct "bands" upon IEF of the CSF. Accordingly, in the serum, where these bands are not observed, the same antibodies should be either absent or, if present, not quantitatively predominant. A direct correlation of the anti-phagotope reactivity with that of antibodies physically present as bands in the CSF has not been attempted because of the insufficient quantity of each CSF sample available for these studies. However, with specific reagents, it is possible to measure the selective enrichment in the CSF typical of intrathecally produced antibodies.

The selected phagotopes have been used as reagents to determine the "specific reactivity" of anti-phagotope CSF and serum antibodies. To this end, equal amounts of serum and CSF IgG from the same patients were tested by ELISA using immobilized phagotopes as ligands. The results, reported in Fig. 5, show that the specific reactivity of the antibodies reacting with phagotopes MS1c, MS17, and MS12 is significantly higher in the CSF than in the serum of patients 1, 38, and 2, respectively, ranging from 14- to 50-fold. The same results were obtained using different quantities of total IgG (data not shown). These data are compatible with the hypothesis that the tested phagotopes react with antibodies produced locally in the CNS.

Different results were obtained when MS17 and MS12 were subjected to the same kind of analysis using CSF and serum pairs from the patients 1 and 64, respectively. In these two cases, serum and CSF showed the same reactivity (Fig. 5), indicating that although antibodies with the same binding specificity can be present in different patients, they are not always enriched in the CSF.

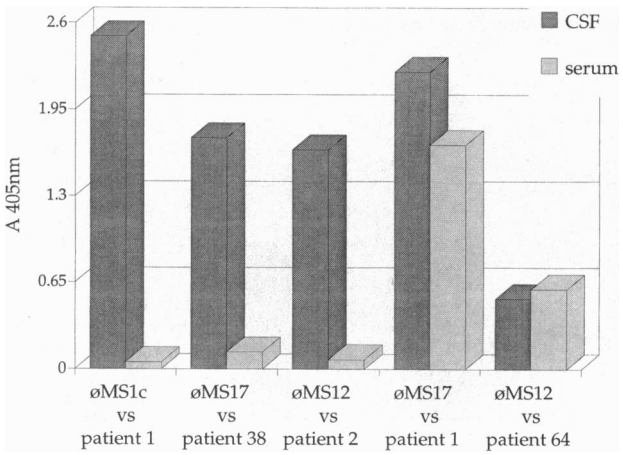

FIG. 5. CSF antibodies recognizing the selected phagotopes represent enriched species. Binding of IgG present in the CSF (darkly shaded histograms) and in the sera (lightly shaded histograms) of MS patients was measured by ELISA. Equal amounts (12.5 ng) of CSF and serum IgG were used. Tested phagotopes and patients' antibodies are indicated at the bottom. Average values from two experiments have been determined. Results are expressed as the difference between the average value of the tested phagotope and wild-type phage (A_{405}).

DISCUSSION

During the onset and development of several diseases, there is a readjustment of the antibody repertoire, reflecting the exposure to novel antigens or the altered equilibrium of the antibody network. A direct analysis of the disease-specific B-cell response might reveal information important to the understanding the etiology and pathogenesis of the disease and to identifying the pathological antigens. However, the characterization of disease-specific antibodies is difficult and incomplete if the pathological antigen is not known. For MS, whether OB and general intrathecally produced antibodies recognize the same antigen, whether they are also present in the serum, and, most importantly, whether they are MS-specific have remained unanswered questions due to the lack of specific reagents. In this paper, we have used RPLs as a source of specific reagents for the characterization of antibodies in the CSF. We established that (*i*) antibodies displaying the same binding specificity are present in the CSF and the serum of the same patient; (*ii*) these antibodies are probably directed against rather ubiquitous antigens; and (*iii*) some of these antibodies are specifically enriched in the CSF. Our results, to be confirmed on a larger sample of patients, also suggest that each MS patient has a different set of specific intrathecally produced antibodies in the CSF.

In previous studies using sera from patients and RPLs, we observed that after one or two rounds of selection, the amino acid sequences of the selected phagotopes were extremely diverse, without any recognizable common motif (R.T. and A.N., unpublished observations). This finding reflects the nature of a polyclonal antibody population in which there is a large number of different antibody species, including those specific to the disease, none of which measurably predominates over the others. By contrast, in this study the selected peptides were not so diverse, suggesting that, in the antibody population of the CSF used for the selections, there are predominant species, possibly corresponding to intrathecally produced antibodies.

Until now, OBs have been identified and defined by comparing the pattern of IgG present in the serum and CSF from the same individual by IEF: an OB is such if it is detected in the CSF but not in the serum. The most likely explanation for the presence of OBs is that they correspond to antibody species selectively enriched in the CSF. By using the CSF-selected phagotopes as reagents in ELISA, we have shown that IgG displaying equivalent specificity are concomitantly present in the CSF and in the serum of the same MS patients and, in some cases, they are considerably more abundant in the former than in the latter. This result corresponds to expectations for OB antibodies and, at the same time, provides for a novel tool for the identification of intrathecally produced antibodies based on a quantitative measurement using specific ligands, rather than a qualitative inspection of the IEF pattern. In some patients, a similar reactivity against the phagotopes is observed in the CSF and the serum. Even in these cases, the reacting species in the CSF could correspond to CSF-enriched antibodies, as the same epitope could be recognized by several different antibodies in the serum, whereas only a subset of these species could be present, and enriched, in the CSF. An alternative explanation is that the abundance of such antibodies in the CSF is due to the passive passage of highly concentrated serum species across the blood–brain barrier.

The distinct pattern of reactivity with CSF from various MS patients and their behavior in cross-competition experiments demonstrate that the three classes of phagotopes mimic different natural epitopes, thus providing experimental evidence that CSF antibodies in MS patients do not have the same specificity. These antibodies are very frequently present in the sera of MS patients and normal controls, suggesting that their target phagotopes mimic rather ubiquitous antigens to which

many individuals are exposed. However, in contrast to their high prevalence in the sera, anti-phagotope antibodies displaying the same specificity are only detected in the CSF of a few MS patients. Therefore, it is unlikely (although not yet ruled out) that antibodies against these ubiquitous antigens play a direct crucial role in the etiology or pathogenesis of MS at the stage of disease in which they were detected.

The CSF-enriched antibodies are most likely the result of local intrathecal synthesis and might at least in part correspond to OBs. The enrichment might be a consequence of a rare stochastic event, the passage of a B cell across the barrier, to reach a microenvironment that is favorable for antigen-independent proliferation. Alternatively, the passage is not necessarily rare, but only those B cells that produce antibodies against antigens present in the CNS proliferate, leading to their enrichment. The second hypothesis is testable because it predicts that CSF-enriched antibodies, including OB antibodies, react with antigens (or self-antigens) present in the CNS. To this end, phagotopes could be used to immunopurify the corresponding antibody species or to raise specific antisera. These antibodies could serve as reagents to reveal the presence of the putative corresponding antigen in CNS tissue preparations from normal and MS patients. The same antibodies could also be useful to identify and characterize the natural antigens, using a strategy that has proved successful for other pathologies (31, 32).

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