Distortion of the Self-Reactive IgG Antibody Repertoire in Multiple Sclerosis as a New Diagnostic Tool[1]

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To date, none of the myelin-associated Ag targets definitively discriminates between the immune response observed in multiple sclerosis (MS) patients and healthy subjects. However, it has been shown recently that analysis of global immune Ab profiles such as natural autoantibody reactivities can help to distinguish between normal individuals and patients suffering from various immune diseases. The aim of our study was to compare the global IgG immune response against brain self-Ags in sera from 82 MS patients and 27 healthy subjects. The analysis of the immune profiles was performed by Western blotting, and data were subjected to linear discriminant analysis. Particular patterns of IgG reactivity were found in healthy subjects, Sjögren patients, and MS patients. Moreover, this approach separated the three clinical forms of MS with a high concordance rate with the clinical data (κ value, 77.8%). Our study suggests, for the first time, that serum IgG Ab repertoires are able to distinguish MS patients. In addition, our data suggest that patterns of IgG reactivity could model the pathological processes underlying the various forms of MS. Further characterization of such discriminant Ags could provide useful information regarding their potent role in pathogenesis or regulatory processes in MS. ***The Journal of Immunology,*** **2004, 172: 669–678.**

Multiple sclerosis (MS)[3] is an inflammatory demyelinating disease of the white matter of the CNS. An autoimmune process, infiltrated by macrophages and mononuclear cells, is postulated to be the underlying mechanism of MS lesions. Due to its similarities with experimental autoimmune encephalomyelitis, MS is thought to be mediated mainly by T cells. However, some recent studies have also suggested that B cell activation, Ab response, and humoral factors are potent effector mechanisms involved in the full development of demyelinating disease (1, 2). However, the molecular targets and the mechanisms involved in myelin damage have yet to be clearly defined (3).

Different self-proteins have been investigated as potent targets for T or B cells. The most extensively studied putative self-Ags are components of normal CNS myelin (myelin basic protein (MBP), proteolipid lipoprotein, myelin oligodendrocyte glycoprotein (MOG), etc.), posttranslationally modified forms of these myelin proteins (4, 5), or components originating from glial cells (6, 7). Immune recognition of intracellular proteins by autoantibodies (2′3′-cyclic nucleotide 3′-phosphohydrolase) has also been described (8). Nevertheless, whether or not there is an alteration of the immune recognition of self-proteins in MS compared with normal subjects requires further investigations.

To date, no specific biological diagnostic marker of MS has been identified. Specific autoantibodies detected in sera from patients are usually investigated by techniques using purified self-Ags and/or relevant peptides from preselected targets. Such a restrictive view may be overcome by using a large panel of Ags derived from target tissue extracts. In the present study, we compared by Western blot the IgG repertoires of healthy subjects and MS patients against a broad range of self-Ags derived from brain homogenates from one MS patient and from three healthy subjects. Our data demonstrate that serum IgG immune profiles can discriminate between healthy subjects and MS patients and can also differentiate the three clinical forms of MS. This approach has led to the identification of new potential antigenic candidates, such as α-enolase. The pathogenic or regulatory role of these discriminant Ags remains to be defined. Such newly identified antigenic targets, serving as markers of disease activity, could be useful diagnostic tools.

Materials and Methods

Patients

IgG Ab response to brain tissues was studied with sera from 128 subjects. Eighty-two patients were diagnosed with clinically definite MS according to the criteria of Poser et al. (9). All patients were followed up in the Department of Neurology (Centre Hospitalier Régional Universitaire, Lille, France) and were relapse free. The sera were analyzed before immunomodulating or immunosuppressive treatment. Sera from 27 healthy subjects were tested as normal controls. Nineteen patients having Sjögren syndrome (SS) with neurological disorders were included as a second control group. Table I summarizes the epidemiological parameters of patients and controls. All subjects gave their written informed consent, and the study was approved by the local ethics committee.

IgG purification

Serum samples were treated by affinity chromatography on protein G-Sepharose column (Amersham Pharmacia Biotech, Uppsala, Sweden). IgG was purified according to the manufacturer's recommendations. Shortly after the binding of the IgG on the matrix, the IgG was eluted using glycine buffer (pH 2.8). The IgG-depleted serum was kept for IgM evaluation. Affinity-purified IgG was then neutralized by adding neutralizing buffer directly to the fraction at column end. To evaluate the appearance of

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[3] Abbreviations used in this paper: MS, multiple sclerosis; MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; SS, Sjögren syndrome; IPG, immobilized pH gradient; IEF, isoelectric focusing; 2-DE, two-dimensional electrophoresis; pI, isolectric point; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; LDA, linear discriminant analysis.

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Table I. *Clinical and demographic characteristics of the MS patients and control subjects*

	n	Mean Age ± SD (years)	Female/Male
MS patients	82	42.3 ± 11.1	42/40
Relapsing-remitting	45	37.4 ± 12.1	28/17
Secondary progressive	15	52.7 ± 10.3	9/6
Primary progressive	22	47.5 ± 9.6	5/17
Healthy subjects	27	32.8 ± 8.28	14/13
SS individuals	19	43.3 ± 7.37	12/7

polyreactivity, a part of the acidic fraction containing the purified IgG was dialyzed against PBS (pH 7.2) as described elsewhere (10).

Antibodies

To characterize reactivities on Western blot, we created landmarks using commercial Abs directed against particular targets. The anti-MBP Ab used was a rat polyclonal Ab (Serotec, Raleigh, NC). A goat anti-human enolase Ab (Santa Cruz Biotechnology, Santa Cruz, CA) was used as a positive control. The second Ab were HRP-conjugated goat anti-rat IgG or rabbit anti-goat IgG (Sigma-Aldrich, St. Louis, MO).

Brain samples

The brain samples, dissected out at autopsy from the frontal lobe in Brodmann's area 10, were obtained from one MS patient (a 56-year-old woman) and one healthy 28-year-old woman with no history of neurological disease (Department of Neuropathology, Centre Hospitalier de l'Université de Lille, and Institut National de la Santé et de la Recherche Médicale, Unité 422, Lille, France). The white matter sample was dissected out from the corpus callosus, obtained from the healthy 28-year-old woman. This woman died as a result of complications during delivery. The autopsies were performed within the framework of a tissue collection program that had been approved by the local ethics committee. The brain sample was dissected out by deep core sampling to obtain all the cortical structure and the subcortical white matter. In each case, the postmortem delay was <8 h. Two other SDS control brain homogenates from autopsy samples were purchased from Clontech (Palo Alto, CA) as well as the kidney, liver, spleen, skeletal muscle, and lung protein homogenates.

SDS-PAGE

The brain samples were homogenized in a Tris buffer containing 5% SDS at a final concentration of 10 mg/ml and heated at 95°C for 10 min; 80 μl of this lysate was loaded per well onto a 10–20% gradient polyacrylamide gel, beside a molecular mass marker (Amersham Pharmacia Biotech). Just before electrophoresis, the homogenates were reduced with 10 mM DTT (Sigma-Aldrich). Electrophoresis was run for 12 h in Laemmli buffer at 100 V (11).

Blotting and analysis procedures

Proteins were transferred onto 0.45-μm ECL nitrocellulose membranes (Amersham Pharmacia Biotech) at 0.8 mA/cm^2 (12) and later saturated with 5% nonfat dried milk. Each well was cut into 15 strips, 3- to 4-mm wide. Western blotting was conducted with total sera, diluted 1/100 in TBS (100 mM Tris (pH 8.0), 0.3 M NaCl) containing 0.5% Tween 20 (w/v) and 5% nonfat dried milk. After incubation for 1 night at 4°C, the IgG Abs were revealed with an anti-human Fcγ HRP-conjugated Ab (1/10,000; Sigma-Aldrich). Fluorograms were prepared with an ECL kit (Amersham Pharmacia Biotech). Immune profiles were analyzed when three independent assays had produced identical patterns. Densitometric analyses were performed on nonsaturated autoradiographs using the Quantity One software (Bio-Rad, Hercules, CA) apparatus to localize and compare the IgG immune profile patterns. The Ab reactivities were superimposed and aligned using Diversity database 2.2 software (Bio-Rad). Two different operators performed the complete analyses of the profiles blindly.

Bidimensionnal electrophoresis

The brain sample was homogenized in a lysis buffer (7 M urea/2 M thiourea (Sigma-Aldrich), 50 mM *N*-octyl glucoside, 1× anti-protease mixture (Sigma-Aldrich), and 1% DTT (Sigma-Aldrich)) at a final concentration of 10 mg/ml. The immobilized pH gradient (IPG) strips (pH 3–10; linear or nonlinear; Immobilines, Amersham Pharmacia Biotech) were rehydrated overnight with a reswelling solution containing 9 M urea, 1% DTT, 4% Triton X-100, and 2% v/v Pharmalytes (Amersham Pharmacia Biotech) (pH 3–10) (13). Sample load was realized by in-gel rehydratation using 0.5–1 mg of protein derived from brain tissue on each IPG strip. Proteins were separated using the MultiPhor II (Amersham Pharmacia Biotech) and Bio-Rad Protean II xi chamber, according to the manufacturers' instructions. Paper wick electrodes were soaked with buffer (anode, 10 mM H_3PO_4; and cathode, 10 mM NaOH) and blotted against filter paper to remove excess buffer. Because of the high voltage used, isoelectric focusing (IEF) was performed under a layer of silicone oil at 20°C (14). For the first dimension, the IEF program was as follows: 150 V, 1 h; 300 V, 1 h; 1000 V, 1 h; and 3500 V until a minimum Vh product of 50 kVh was reached. After termination, the IPG strips were stored at −70°C until further use or directly equilibrated for 3 × 30 min in 3 × 2-ml equilibration solution (50 mM Tris-HCl (pH 8.8), 8 mM EDTA, 10% w/v glycerol, 5% w/v SDS, and 1% w/v DTT). Equilibrated IPGs were transferred to a polyacrylamide gradient gel (T = 9–16%) containing piperazine diacrylamide (C = 2.6%; Bio-Rad) (15). Gels were polymerized overnight. Electrophoresis was run for 14–16 h with current limited to 40 mA/gel. Gels were stained with Coomassie brillant blue G-250 (Sigma-Aldrich) or with silver nitrate (Invitrogen, San Diego, CA). For Western blotting, the gels were electroblotted onto polyvinylidene difluoride membranes (Amersham Pharmacia Biotech). The IEF and the two-dimensional PAGE experiments were repeated four times.

For immunostaining, two-dimensional electrophoresis (2-DE) gels were transferred onto polyvinylidene difluoride membranes and treated as described earlier.

Immune profiles were analyzed when two independent assays had produced identical patterns. The molecular mass was determined by comparison with standard protein markers (low-molecular-weight standard; Amersham Pharmacia Biotech), covering a range of 14.4–97 kDa, at the right side of selected gels. The isolectric point (pI) values were estimated using carbonic anhydrase pI marker (Bio-Rad) and standard curves for pH gradient visualization provided by the supplier of the IPG strips. The two-dimensional protein patterns in the gels and autoradiographs were analyzed with the PDQuest software (Bio-Rad). A series of spots were recognized by the majority of subjects and were used for internal calibration to superimpose gels and Western blot images.

In-gel digestion and matrix-assisted laser desorption ionization-time of flight (MALDI-TOF)-mass spectrometry analysis

Excised plugs from Coomassie-stained gels were destained with 200 μl of 50% acetonitrile in 10 mM NH_4HCO_3 and dried in a SpeedVac concentrator. Protein was digested overnight at 37°C by sequencing-grade trypsin (5 μg/ml; Promega, Madison, WI) in 50 mM NH_4HCO_3. The resulting peptides were extracted twice with 25 μl of 50% acetonitrile/0.1% trifluoroacetic acid. The collected extracts were lyophilized, and were resuspended in 10 μl of 0.1% trifluoroacetic acid and desalted on ZipTip C18-microcolumns (Millipore, Bedford, MA). Elution was performed with saturated α-cyano-4-hydroxycinnamic acid directly onto the MALDI target (2 μl of the solution were applied to a plated sample holder and introduced into the mass spectrometer after drying). MALDI-TOF-mass spectrometry was used to obtain mass fingerprinting for proteins using a Voyager DE-SIR instrument (Applied Biosystems, Framingham, MA). Ions were accelerated at 20 kV and reflected at 21.3 kV. Spectra were acquired in the delayed extraction, reflectron R mode. A total of 100–300 scans was averaged to produce final spectra. Spectra were externally calibrated using the monoisotopic MH+ ion from four peptide standards (trypsin autodigestion products).

Database search based on peptide mass fingerprint spectra

The obtained peptide mass fingerprint spectra were analyzed by searching the National Center for Biotechnology Information nonredundant protein database with ProFound (http://prowl.rockefeller.edu/cgi-bin/ProFound), version 3.2, and Peptident programs. The parameters for each search were varied to achieve the best results. The standard parameters were established as follow: *Homo sapiens*, 0- to 250-kDa molecular mass (depending on the region where the spot occurred in gel), tryptic digest with a maximum number of one missed cleavage. Peptide masses were stated to be monoisotopic, and methionine residues were assumed to be partially oxidized. The mass tolerance was set to 0.1 Da after internal calibration using three trypsic self-peptides of 842.510, 1045.564, and 2211.1046 Da.

Statistical analysis

The data were expressed in binary mode (0, absence of antigenic band; 1, presence of an antigenic band) to subject IgG Ab patterns to analysis using

either the $\div^2$ or Fisher exact test. This approach allowed us to select the most relevant Ags that support qualitatively different immune recognition, both between healthy subjects and MS patients and between the different clinical forms of MS.

In a second stage, we used linear discriminant analysis (LDA) to balance the discriminating Ags between the populations of individuals. All the Ags having a p value <0.2 in the previously used statistical tests were selected for LDA (16). Using a stepwise logistic regression model and supported by the LDA method, we were able to isolate a subgroup of brain Ags related to their strength of discrimination between the different populations studied.

By associating two parameters, for the presence ($\times 1$) or absence ($\times 0$) of each selected Ag, and the coefficient previously defined by the LDA, a score was calculated for each subject as a representative value of the individual immune profile, using the following formula: score = $Ag1_{coef1} \times (0_{(absent)}$ or $1_{(present)}) + Ag2_{coef2} \times (0_{(absent)}$ or $1_{(present)}) + Ag3_{coef3} \cdot \ldots$ Statisticians calculated all of the scores blindly. The calculated scores were graphically projected on an $(n - 1)$ axis, where n is the number of groups included in the LDA. First, the analysis focused on discrimination between MS patients and controls (healthy subjects and SS patients). The threshold values were determined using a receiver operating characteristic curve. The positive and negative predictive values were also calculated. In a second stage, the analysis focused on the different clinical forms of MS. A κ test was used to evaluate the concordance with clinical data.

Results

Ab IgG repertoires against brain tissues in MS patients

The IgG Ab response of 82 sera collected from MS patients was tested successively against control brain tissue and MS brain tissue using an immunoblotting assay. An analysis of the different patterns obtained with regard to the molecular mass of the proteins recognized by IgG Abs revealed the presence of 4–32 bands (mean ± SD, 18 ± 4 bands) per strip and per tissue for each MS serum tested.

Quite different patterns of recognition were found when MS sera were tested against either control brain tissue or MS brain homogenate (Fig. 1*A*). Different patterns were also noted when a given serum was tested successively against control brain tissue and MS brain tissue, as revealed by densitometric analysis of the profiles. In addition to qualitative variations, the densitometric analysis of immune profiles obtained with the 82 sera also revealed interindividual differences in the level of self-reactivity against brain Ags with MS sera (Fig. 1*B*).

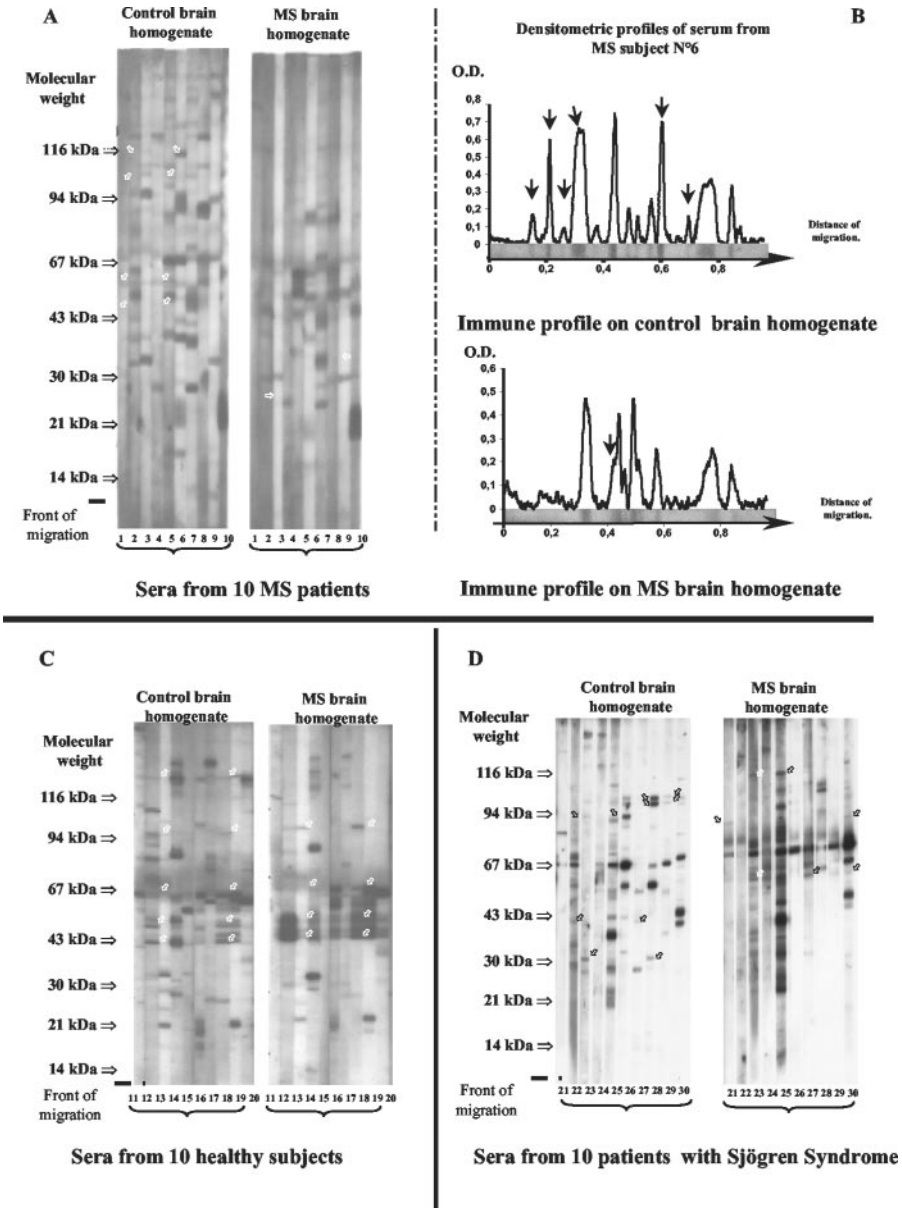


FIGURE 1. Immunoreactive patterns with sera from healthy subjects, MS and SS patients against brain Ag. IgG immune profiles obtained with sera collected from 10 MS patients (*A* and *B*), 10 healthy subjects (*C*), and 10 SS patients (*D*), were tested against control and MS brain homogenates. As revealed by Western blotting, different patterns were observed when each serum (numbered 1–10, 11–20, and 21–30 for MS, healthy, and SS subjects, respectively) was tested against the same brain tissue (control or MS). All of these IgG reactivities showed a high diversity of immune recognition among the three groups tested with regard to the number and the nature of protein bands recognized. Nevertheless, some similar antigenic bands were detected with distinct sera incubated either against control brain homogenate or against MS brain homogenate, as illustrated by open arrows. *B*, Shown are representative densitometric profiles of IgG reactivity for one MS patient.

Despite a high degree of heterogeneity with regard to the number and the nature of the protein bands recognized, a more detailed analysis of the patterns enabled us to detect partial common clusters of protein bands with distinct MS sera in control brain tissue as well as in MS brain homogenate (Fig. 1*A*).

Ab IgG repertoires against brain Ags in healthy subjects

The IgG Ab response of 27 normal sera against control brain and MS brain homogenates was also evaluated by immunoblotting. An analysis of the different patterns with regard to the molecular mass of the proteins recognized by IgG Ab showed that 8–29 bands (mean ± SD, 18 ± 5 bands) per strip and per homogenate could be detected for each normal serum.

As previously observed with MS sera, quite different patterns of recognition were found when normal sera were tested against control brain tissue or against MS brain tissue (Fig. 1*C*). As observed for MS sera, the profiles obtained with the 27 normal sera revealed qualitative and quantitative interindividual differences in terms of self-reactivity (data not shown).

A more detailed analysis of the patterns enabled the detection of partial common clusters of antigenic bands with distinct normal sera. Indeed, IgG Abs from different sera recognized some similar bands among all the immunoreactive antigenic bands in control brain homogenate as well as in MS brain tissue (Fig. 1*C*).

Self-reactive IgG repertoire against brain Ags in SS patients

As described with MS and healthy subjects, the IgG Ab response of 19 SS sera against control brain and MS brain homogenates was evaluated by immunoblotting assay (Fig. 1*D*). In this case, the analysis of the different patterns with regard to the molecular mass of the proteins recognized by IgG Ab showed that 3–32 bands (mean ± SD, 16 ± 7 bands) per strip and per homogenate could be detected for each SS serum. The comparison of patterns obtained with MS brain and with control brain tissues revealed a high degree of diversity of IgG response in SS sera with interindividual variations in IgG profiles. Nevertheless, focusing on particular bands, we showed that some antigenic recognitions appear to be common among different sera (Fig. 1*D*).

Relevance of immunoblotting for evaluation of IgG Ab repertoire analysis

Variable patterns of IgG immune recognition were found both with normal and MS sera against control or MS brain tissues. To determine whether this high degree of heterogeneity was evidence of a singular immunological status for each subject, different control tests were performed.

To assess the reproducibility of the method used, the same MS serum was successively tested against three distinct control brain homogenates. As shown in Fig. 2*A*, similar IgG Ab repertoires were obtained. Same results were also found when a normal serum was tested against the three distinct brain homogenates (data not shown).

Different control tests were also performed to estimate the patterns obtained with the secondary Ab alone. In addition, self-reactive Ab repertoires with whole sera or purified IgG or IgG-depleted sera were also evaluated. As expected, a slight reactivity was noted by using anti-IgG as a secondary Ab without sera preincubation (Fig. 2*B, lane A*). To assess possible interactions between IgG Ab repertoires and autoregulatory anti-idiotypic Abs of IgM isotypes, comparative analyses using whole sera and affinity-purified IgG and IgG-depleted sera were conducted. When anti-IgG was used as a secondary Ab, quite similar patterns were found with whole sera compared with purified IgG (Fig. 2*B, lanes B* and *C*). In this latter case, neutralization of purified IgG was directly performed at the column end (Fig. 2*B, lane C*). In contrast, polyreactivity was observed after an overnight dialysis of eluted IgG as shown in Fig. 2*B, lane D*. Analysis of self-reactive IgM Ab repertoires with whole sera or IgG-depleted sera revealed similar and faint antigenic recognitions.

To assess whether the brain tissue used was suitable for our analysis, immunoblotting assays using a commercial Ab directed against different isoforms of MBP were performed. As shown in Fig. 2*C*, the brain samples, characterized according to Brodmann's classification of cortical areas, also contained subcortical materials as revealed by the presence of major myelin proteins such as MBP.

To assess the singularity of patterns obtained with brain Ags, other human tissues were tested using the same immunoblotting procedures. As shown in Fig. 2*D*, the number and the nature of the protein bands recognized in other tissue extracts were quite different from those obtained with brain tissues, when the same MS serum was tested. Similar results were noted when a normal serum was tested against these tissues (data not shown). For ovary, placenta, testis, skeletal muscle, and lung protein extracts, the patterns of IgG immune recognition was restricted to a few antigenic bands, distinct from those observed with MS and control brain homogenates. For liver, spleen, and kidney protein extracts, the patterns were less restricted but the antigenic bands observed did not comigrate with the antigenic proteins recognized in MS or control brain homogenates.

Identification of discriminant antigenic bands

Respective mapping and alignment of the patterns obtained with the 27 normal sera against both control and MS brain tissues allowed us to identify 145 antigenic bands, ranging from 8 to 180 kDa. With the 82 MS sera, 149 antigenic bands were found, ranging from 10 to 160 kDa. Using the SS sera, a total of 110 antigenic bands, ranging from 15 to 130 kDa, were found. On account of the presence of some similar bands, a total number of 162 antigenic bands were identified, as shown in Table II. In normal brain tissue, 87 antigenic bands were found, whatever the source of sera (T1 to T79). In MS brain tissue, 75 antigenic bands were found, whatever the source of sera (S1 to S72). As shown in Table II, χ^2 analysis and Fisher exact tests allowed us to distinguish antigenic bands significantly linked either to MS disease or to controls (healthy status and SS). Of the 15 antigenic bands shown to distinguish MS patients and control subjects, 8 were present in control brain tissue and 7 were present in MS brain tissue. The χ^2 and Fisher analyses also revealed 9 antigenic bands that were differentially recognized by the distinct clinical forms of MS (Table II). Of these 9 antigenic bands, 7 were present in control brain tissue and 2 in MS brain tissue.

Retrospectively, such analyses allowed us to associate antigenic bands with clinical forms of MS as defined by clinical data. Thus, band T56 was more specifically found with sera collected from patients with relapsing-remitting MS, whereas band S72 was principally found with sera from patients with secondary progressive MS. Bands T9 and T61 were associated with secondary progressive and relapsing-remitting MS. Bands T43 and T46 were associated with primary progressive MS and more rarely with secondary progressive MS. Bands S56, S58, and T75 were associated with secondary progressive and primary progressive MS.

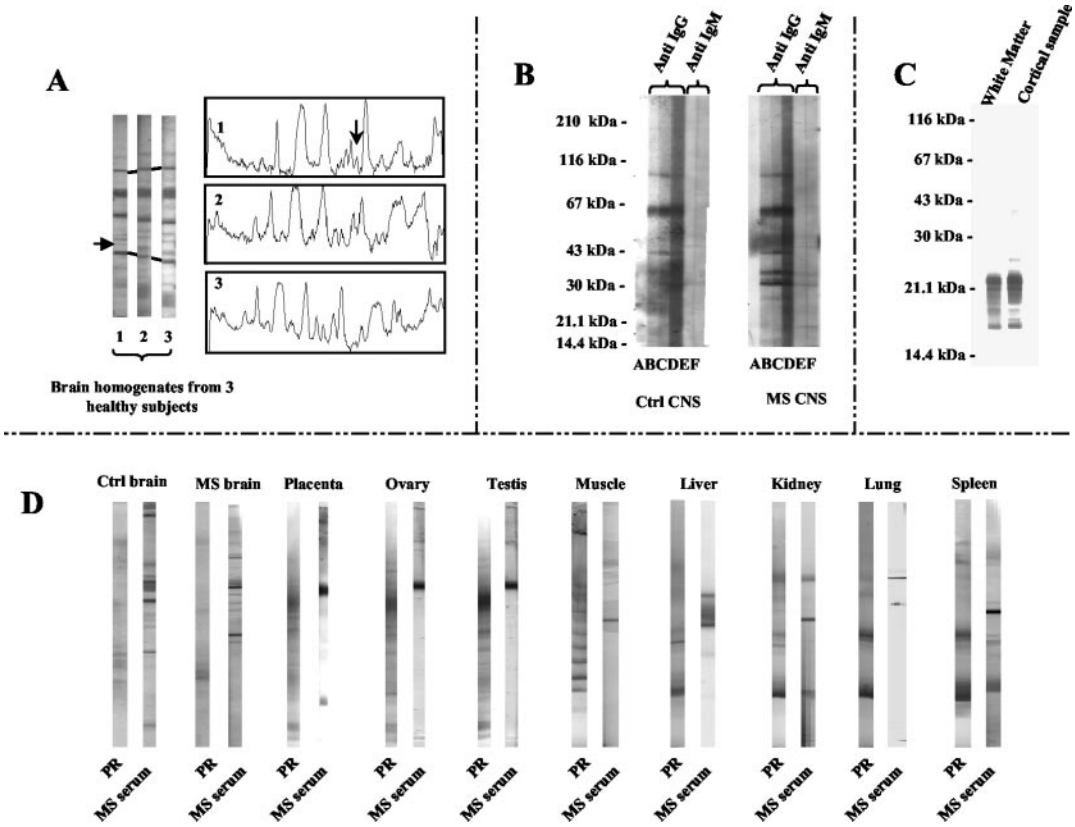


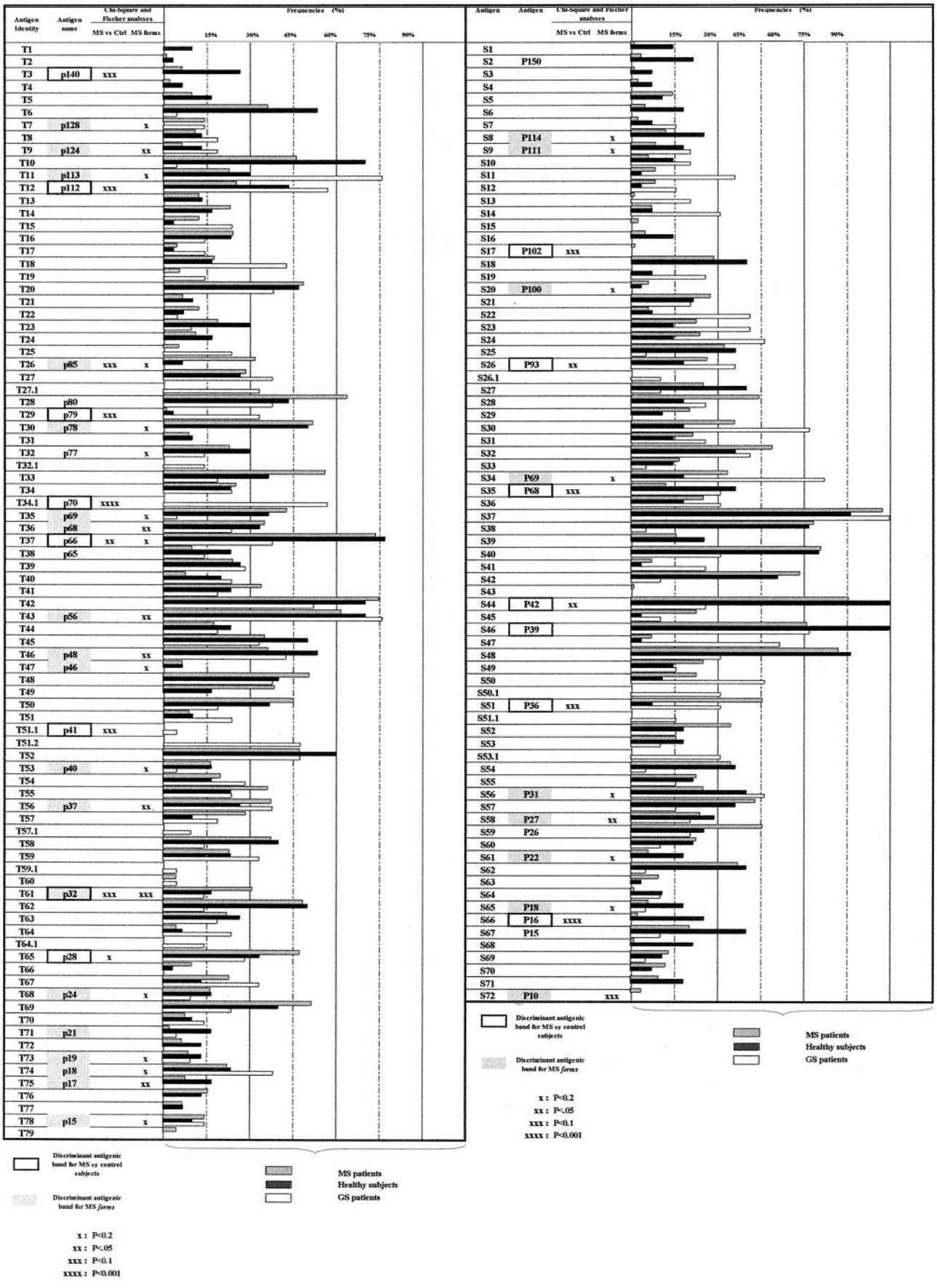
FIGURE 2. Immune profiles characteristics. *A*, Sera from the same MS patient was successively tested against three distinct brain homogenates derived from different autopsy samples collected from three subjects with no history of neurological and/or immunological disease: a 55-year-old woman (*1*), a 65-year-old woman (*2*), and a 28-year-old woman (*3*). Although we observed some minor migration defects, similar patterns of immune reactivity were found. Taking into account the electrophoresis bias, similar densitometric profiles were also found. The small black arrow indicates a minor variation. *B*, The different patterns using either anti-IgG or anti-IgM as secondary Abs are shown as indicated in the *top* of the lanes. *Lane A*, The results obtained with the anti-IgG alone are shown. *Lanes B—F*, Evaluations were performed by using the same MS serum. Reactivity of Abs in whole serum revealed by anti-IgG (*lane B*); reactivity of Abs with purified IgG after chromatographic separation when neutralization of purified IgG was performed at column-end (*lane C*); polyreactivity of Abs with purified IgG after chromatographic separation when neutralization was conducted by overnight dialysis (*lane D*); reactivity of Abs in whole serum revealed by anti-IgM (*lane E*); and reactivity of Abs in IgG-depleted serum (*lane F*). *C*, Reactivity of anti-MBP Abs was evaluated against white matter as well as against brain cortical sample. The Ab recognized an epitope present on each isoform of MBP. *D*, The patterns obtained with brain homogenate and other tissues, as indicated at the *top* of the lanes, were compared by using the same MS serum. For each test, beside the global protein pattern observed after membrane staining with Ponceau red (PR), Ab reactivity of the MS serum is shown.

Relationships between the IgG immune profiles and the clinical status

The above statistical approaches identified antigenic bands recognized by sera of controls subjects (SS patients and healthy individuals) and/or patients with different clinical forms of MS. The relevance of such data was further studied using LDA to take into account a more global immune profile to more accurately distinguish the different populations studied. Some antigenic bands, described in Table II, showed different IgG immune reactivities between MS patients and healthy subjects ($p < 0.2$). These bands were selected to perform an LDA, as previously described (17). Although single antigenic bands with $p < 0.2$ only indicate tendencies, several of them, associated by the LDA, lead us to differentiate more accurately the different populations tested (18). First, we applied this approach to distinguish between control subjects and MS patients (Fig. 3). The LDA enabled us to distinguish 16 antigenic bands (Fig. 3*A*). Nine of these bands were present in control brain homogenate and 7 in MS brain tissue. Coefficient values assigned by LDA for each Ag associated with the presence or the absence of each discriminant Ag enabled the calculation of graphic coordinates for each individual as shown in Fig. 3*B*. Our analysis differentiated between MS patients and control subjects on a two-axis graph projection system (Fig. 3*B*). A receiver operating characteristic curve delineated a threshold value of 0.750 (data not shown) that differentiated MS patients from healthy subjects with a sensitivity of 96.3% and a specificity of 88.9%. A threshold value of 0.3 distinguished SS patients from the others individuals with a sensitivity of 100% and a specificity of 100%. The results showed an excellent degree of concordance with clinical data ($\kappa = 0.92$).

We used a similar approach to study profiles within the MS group to try to separate the different clinical forms. An LDA taking into account protein bands selected by χ^2 analysis or Fisher test revealed 29 antigenic bands (Fig. 4*A*). Twenty bands were present in control brain, and 9 bands were present in MS brain. The LDA defined a two-equation system that projected each case studied onto a two-axis graph (Fig. 4*B*). Threshold values at 1.4 on the *x*-axis, and at 0 at the *y*-axis delineate three areas. Area I contains 41 of 46 (89.2%) MS patients with relapsing-remitting MS. Area II contains 12 of 14 (85.7%) patients with secondary progressive MS. Area III contains 20 of 22 (90%) patients with primary progressive MS. The results obtained showed an excellent degree of concordance with clinical data ($\kappa = 0.778$).

Table II. *Statistical analysis of the IgG reactivity against brain Ags*



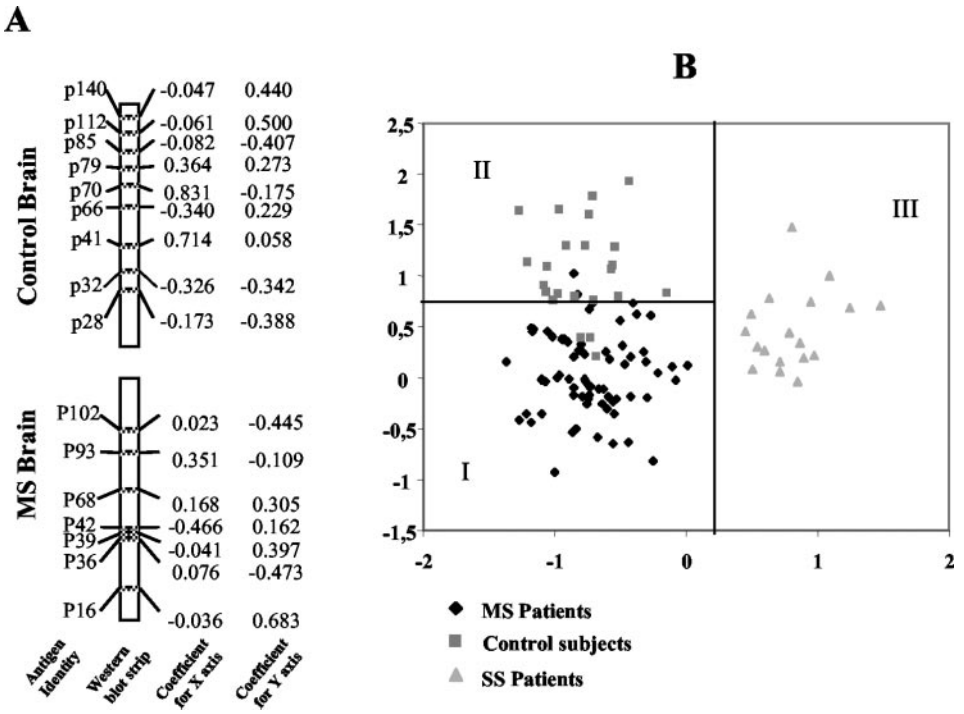


FIGURE 3. LDA distinguishes between the IgG immune profiles of MS patients and control subjects. *A*, Two schematic illustrative strips of Western blot depict the brain Ags that support discriminant immune reactivity on both control and MS brain homogenates with sera from MS patients and from control groups (healthy and SS). The coefficient values assigned by LDA (as described in *Materials and Methods*) were specified for each Ag. *B*, These coefficient values, associated with the presence or absence of each discriminant Ag, led us to calculate graphic coordinates for each individual. The graph clearly shows that the calculated scores of each subject are distributed in three areas according to the clinical status, with an excellent degree of concordance with clinical data ($\kappa = 0.92$).

Characterization of a discriminant Ag

To further characterize discriminant Ags, a proteomic approach was adopted. First, MS sera were used to identify antigenic candidates. Eighteen sera able to recognize all protein bands previously defined as discriminant either in control brain or MS brain in 1-DE were selected. 2-DE followed by immunoblotting assays revealed the presence of multiple antigenic spots. Such heterogeneity in 2-DE was comparable to that observed in 1-DE. The superposition of antigenic spots and protein spots revealed by a standard colloidal Coomassie blue-stained 2-DE enabled the selection of proteins for further in-gel digestion and MALDI-TOF analysis.

Representative antigenic spots are shown in Fig. 5, which illustrates the presence of 29 spots with one MS serum. They were matched on a preparative gel for further characterization with MALDI-TOF as previously described, on the basis of peptide mass matching (19). Such an approach allowed us to identify enolase-α

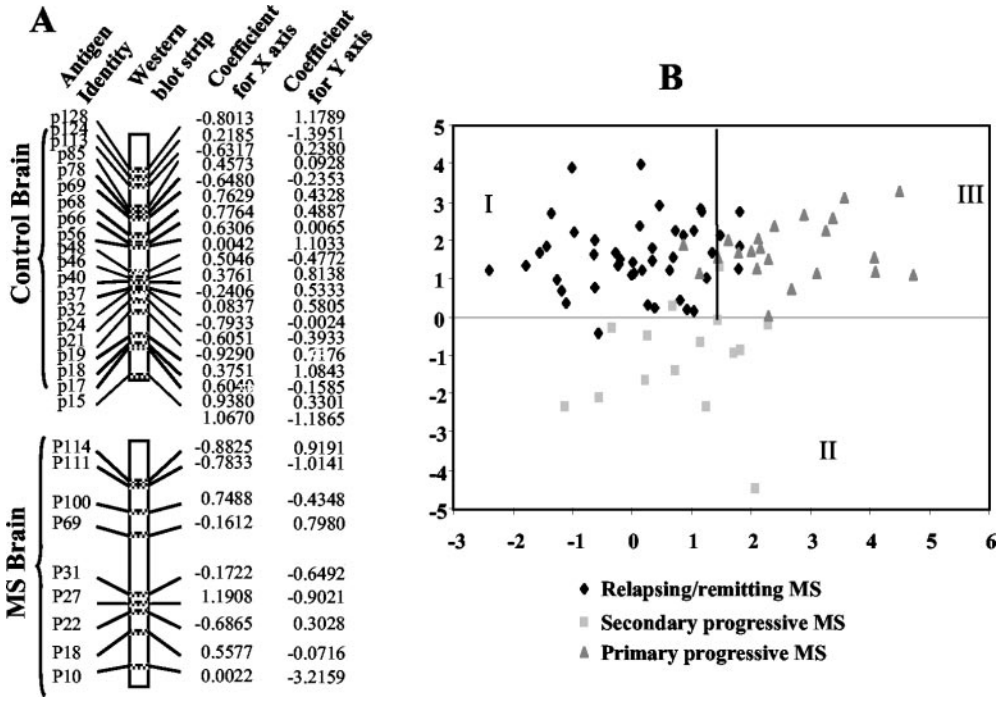


FIGURE 4. LDA separates the different clinical forms of MS. *A*, Two schematic illustrative strips of Western blot depict the brain Ags that support discriminant immune reactivity on both control and MS homogenates in the different clinical forms of MS. The coefficient values for each axis, assigned by LDA, are specified for each Ag. *B*, These coefficient values, associated with the presence or absence of each discriminant Ag, led us to calculate graphic coordinates for each MS patient. A comparison of individual scores with clinical data shows an excellent degree of concordance ($\kappa = 0.778$).

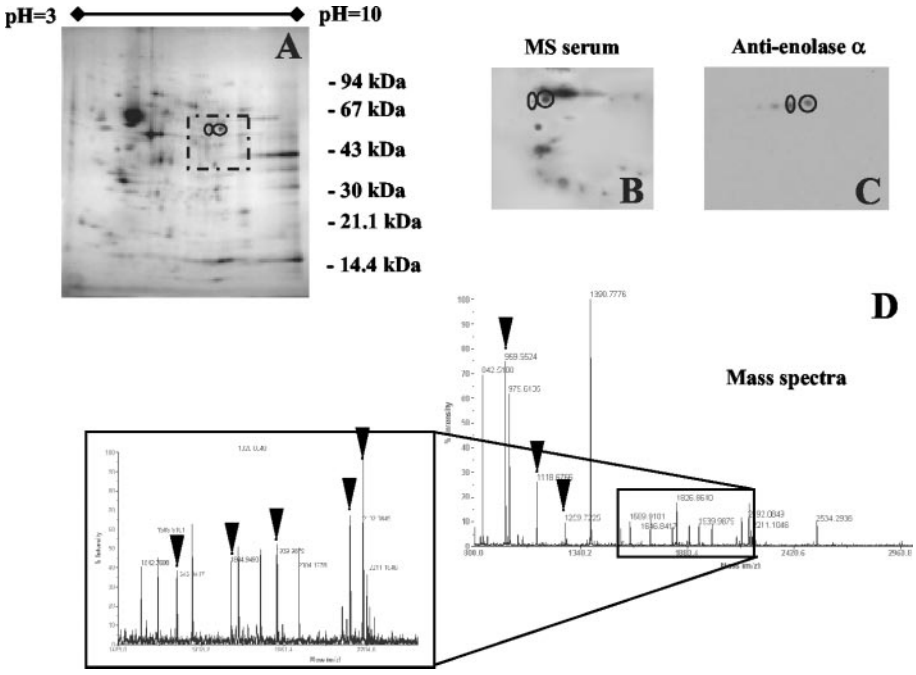


FIGURE 5. Two-dimensional PAGE detection of brain proteins recognized by IgG Abs. *A*, Two-dimensional map of control human brain proteins. The brain proteins from the frontal lobe in Brodmann's area 10 were resolved by IEF on the IPG (pH range, 3–10; linear) and with a 9–16% gradient gel stained with Coomassie brillant blue G250. Molecular mass standards are shown on the *right*. *B* and *C*, Two-dimensional immunoblot was probed either with MS serum (*B*) or with the standard anti-enolase Ab as a control (*C*). The circled antigenic spot represents an MS-specific reactivity. This spot, located recurrently on the gel, was then analyzed by MALDI-TOF-mass spectrometry, following in-gel digestion with trypsin. *D*, The resulting spectra were used for database searching. In this case, they were related to α-enolase (SWISS-PROT accession no. P06733). The arrows indicate the nine peptides matching with the peptide map of α-enolase, leading to a sequence coverage of 30%. The standard anti-enolase Ab recognized the four isoforms of enolase-α (52 kDa; pI, 6.6–6.9). *C*, The different isoforms exhibit the same molecular mass but different pI, suggesting posttranslational modification. *B*, The MS patient serum reacts with the two basic isoforms of the same 52-kDa Ag.

(T43) as a potent discriminant Ag using the SWISS-PROT database (Fig. 5*D*). The use of standard anti-enolase Ab confirmed previous data (Fig. 5*C*).

Discussion

The exact role of physiological self-reactivity in the regulation of the immune response has yet to be determined. The possible involvement of natural autoantibodies has been postulated. These are able to recognize a wide spectrum of self-Ags such as brain Ags (20). Although some interindividual differences in autoimmune reactivity are found in normal sera, several studies have demonstrated a consensual immune pattern of self-recognition defined as an immunological homunculus (18, 20–24). These studies reported the invariance of this immunological homunculus from neonatal to adult status (22, 25). However, when an immunological defect is noted, such as in the hyper-IgM syndrome, a modified pattern of Ab response to self-Ags is found (26). Such data suggest that a distortion of the normal Ab response to self-Ags might be associated with particular pathological processes.

In the present study, we evaluated such a distortion by comparative analysis of the self-reactive Ab repertoires of healthy subjects and MS patients against brain Ags. As previously described (17, 18), we used a Western blotting assay to develop a global approach against a large panel of self-Ags. The patterns of autoimmune recognition against brain homogenates were evaluated with regard to the IgG isotype and by using unfractionated sera. IgG autoreactivity appears to be more closely linked to a pathological process related to autoimmune diseases with isotype switching, which involves the cooperation of T-B lymphocytes and the cytokine network (27–29), even if recent published data suggest an implication of IgM Abs in the MS course (30). In addition, a study with unfractionated sera could more reliably express the net result of autoreactivity, because potent neutralizing factors, such as IgM Abs, are present in normal sera (18). Previous studies have shown that IgM autoantibodies can regulate the natural autoantibody activity of IgG through idiotypic complementarity. Purified IgM could also modulate autoimmune disorders (31). Nevertheless, our results show clearly that IgM Abs do not neutralize or disturb IgG response against brain Ags. The polyreactivity observed by others (10, 32) with purified IgG after affinity chromatography is probably due to altered binding sites at low pH (33). This latter phenomenon was confirmed by our observation. Indeed, purification of IgG with column-end neutralization did not lead to the polyreactivity. In contrast, such polyreactivity was found with IgG neutralized by overnight dialysis.

In a second stage, normal sera were tested against various tissues (normal brain, MS brain, and other tissues). Antigenic protein bands were detected when normal sera were tested against normal brain homogenate. Such findings suggest the presence of natural IgG Abs which might be involved in a tolerogenic process. A high degree of heterogeneity with regard to the number and the nature of protein bands recognized was evidence of singular IgG Ab repertoires as previously described (18, 34). For the same normal serum, comparative studies between patterns obtained with normal or MS brain revealed a strong variability. The progression of chronic inflammatory processes alters the damaged tissues that can induce the expression of new self-Ags (35) recognized by natural Abs which may be involved in protective processes. Immune mechanisms for reducing the spread of damage and for enhancing

remyelination and tissue reparation and maintaining injured nervous tissues have been previously described (36–38). As previously demonstrated, singular patterns of IgG reactivity were found when results obtained with brain tissues were compared with those obtained with other human tissues. Singular patterns were also observed with MS sera. Nevertheless, MS IgG Ab repertoires are quite different from normal IgG Ab repertoires. To more accurately determine the significance of such differences, further comparative analyses were performed.

To date, routine demonstrative biological testing has been limited to the detection of IgG oligoclonal bands in cerebrospinal fluid, found in ~90% of MS patients (39). It would be of interest to develop new biological markers of MS disease. In this respect, the analysis of self-reactive Ab repertoires using LDA might be informative, as previously shown in Guillain-Barré syndrome (40) and Tourette's syndrome (41). Such analyses were also performed in other diseases. Heterogeneous and broadly altered IgM autoimmune patterns against a panel of whole-tissue extracts as sources of self-Ags have been reported in glomerulonephritis, warm autoimmune hemolytic anemia, and systemic lupus erythematosus (18, 42). Such immune profiles distinguished patients from healthy subjects. However, no representative pattern of immune recognition could be associated with these pathophysiological processes. By contrast, patients with myasthenia gravis share similar immune recognition patterns of both purified IgM and IgG to self-Ags extracted only from the target tissues (thymus and muscle) (43, 44). These data suggest that organ-specific autoimmune diseases might be associated with an alteration of immune recognition of target organs (18). In our study, using LDA, we also found a particular pattern of IgG immune recognition against brain self-Ags when control sera and sera of MS patients were tested successively against normal and MS brain homogenates. The analyses of the respective immune profiles allowed us to differentiate between the three groups with high sensitivities and specificities. Moreover, specific patterns of IgG reactivity also allowed us to distinguish between the three forms of MS with an excellent degree of concordance with clinical data. Thus, we defined 16 brain Ags (9 in normal control brain tissue and 7 in MS brain tissue) that showed a discriminant IgG response between MS and healthy subjects. The differences of patterns between MS patients and control subjects and more especially healthy individuals might be related, as discussed above, to the loss of some natural autoreactivity and the gain of some new self-Ag immune recognition. In line with a proposal made by Tuohy et al. (45), autoimmune diseases might be associated with a spontaneous regression of the primordial autoreactive repertoire, with a shifting and spreading of autoreactivity to new self-determinants—epitope and Ag spreading. Twenty-nine Ags (20 in normal control brain tissue and 9 in MS brain tissue) were identified when the three clinical forms of MS were compared. Of these discriminant antigenic bands, only a few are common when the results of the different LDA are taken into account. A distorted self-reactive IgG Ab repertoire might be representative of each MS subset. It could illustrate the active participation of IgG Ab response in the development of MS lesions. However, it could be evidence of a passive modeling of IgG reactivity related to newly expressed Ags in MS lesions as suggested by experimental observations. In Theiler's murine models, described as a representative model of the primary progressive form of MS, histological studies show that apoptosis of oligodendrocytes are a major event, whereas humoral immune involvement seems to be minor (46).

Among the major discriminant antigenic candidates, α-enolase was identified as a potent indicative antigenic target in MS. Indeed, in the stepwise analysis performed with the LDA, apart from the Ag S72, the major informative antigenic band that distinguished the MS forms was the T43. A proteomic analysis allowed us to characterize as enolase-α the protein involved in this reactivity at 52-kDa apparent molecular mass. This glycolytic enzyme (2-phospho-D-glycerate hydrolase; Enzyme Commission no. 4.1.1.1) is one of the three highly homologous isoenzymes of enolase. The α isoenzyme is present in most tissues, whereas the β form is essentially located in muscle. The γ form is specifically found in nervous tissue. Recently, α-enolase was involved as a novel autoantigen in Hashimoto's thyroiditis-associated encephalopathy (47) and in arthritis rheumatism (48). In our work, it appears as a major antigenic target that supports reactivity to distinguish MS forms. As described by Ochi et al. (47), we observed reactivity only against the basic isoforms, without cross-reactivity against the γ-isoenzyme despite the 83% homology between the amino acid sequences among the three isoenzymes. This result suggests that Abs may react with particular epitopes on this protein (47, 48). Further biochemical and immunological analysis will be needed with respect to specific epitope characterization.

Despite the use of a high-performance method, our analysis of IgG reactivity to brain Ags remains restricted. The Western blot method limits the analysis of Ab response to proteins having a sufficiently high threshold of expression. Furthermore, native conformational epitopes could disappear during the electrophoresis steps, even if partial refolding of proteins is possible (49, 50). This could explain a partial loss of IgG reactivity, and the emergence of a restricted repertoire against self-Ags in sera of both healthy subjects and MS patients. In the same way, this modification of protein conformation might explain the minor reactivities that we have observed with the IgM against brain Ags. By contrast with a recent study that used purified and recombinant proteins as target Ags (30), the absence of reactivity against myelin proteins observed in our study was probably due to the weak representation of these proteins in whole brain tissue samples. In addition, SDS extraction allowed the solubilization of MBP and a large proportion of MOG, but without organic extraction, proteolipid lipoprotein is faintly represented (51–53). Nevertheless, our method suggests that various Ags support specific immune recognition in MS. Separately, protein bands appear as poor markers of disease. In contrast, the combination of IgG response to several brain Ags is more indicative. Interestingly, Berger et al. (30) have shown that the analysis of IgM reactivities against MOG coupled with the search of anti-MBP Abs could be a useful tool to predict an early conversion to clinically definite MS after a first demyelination. Our data highlighted some new targets. Further characterizations are now under investigation by proteomic analysis to more accurately determine their pathogenic and regulatory roles. Their indicative value as biological markers useful for MS diagnosis would then be corroborated on the broadest population by using homemade proteo-chips with discriminant proteins synthesized in vitro.

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