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Recognition and Degradation of Myelin Basic Protein Peptides by Serum Autoantibodies: Novel Biomarker for Multiple Sclerosis

Alexey A. Belogurov, Jr.,* Inna N. Kurkova,* Alain Friboulet,† Daniel Thomas,¶ Viktor K. Misikov,§ Maria Yu. Zakharaeva,* Sergey V. Suchkov,§ Sergey V. Kotov,§ Alexander I. Alehin,§ Bérangère Avalle,¶ Ekaterina A. Sousova,* Herbert C. Morse III,‖ Alexander G. Gabibov,2*† and Natalia A. Ponomarenko*

The pathologic role of autoantibodies in autoimmune disease is widely accepted. Recently, we reported that anti-myelin basic protein (MBP) serum Abs from multiple sclerosis (MS) patients exhibit proteolytic activity toward the autoantigen. The aim of this study is to determine MBP epitopes specific for the autoantibodies in MS and compare these data with those from other neuronal disorders (OND), leading to the generation of new diagnostic and prognostic criteria. We constructed a MBP-derived recombinant “epiplate library” covering the entire molecule. We used ELISA and PAGE/surface-enhanced laser desorption/ionization mass spectroscopy assays to define the epitope binding/cleaving activities of autoantibodies isolated from the sera of 26 MS patients, 22 OND patients, and 11 healthy individuals. The levels of autoantibodies to MBP fragments 48–70 and 85–170 as well as to whole MBP and myelin oligodendrocyte glycoprotein molecules were significantly higher in the sera of MS patients than in those of healthy donors. In contrast, selective reactivity to the two MBP fragments 43–68 and 146–170 distinguished the OND and MS patients. Patients with MS (77% of progressive and 85% of relapsing-remitting) but only 9% of patients with OND and no healthy donors were positive for catalysis, showing pronounced epitope specificity to the encephalitogenic MBP peptide 81–103. This peptide retained its substrate properties when flanked with two fluorescent proteins, providing a novel fluorescent resonance energy transfer approach for MS studies. Thus, anti-MBP autoantibody-mediated, epitope-specific binding and cleavage may be regarded as a specific characteristic of MS compared with OND and healthy donors and may serve as an additional biomarker of disease progression. The Journal of Immunology, 2008, 180: 1258–1267.

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the human CNS with heterogeneous pathophysiological and clinical manifestations and a very complicated etiology (1, 2). The viral mimicry hypothesis was formulated to explain the initiation of this pathology (3). At present, however, the true triggering mechanisms of the disease have not been clearly defined (4). Despite strong evidence for T cell involvement in MS in humans and experimental animal models of the disease, the contributions of a specific B cell response to myelin sheath destruction also deserves consideration (1, 5, 6). Direct high-resolution microscopic analyses revealed the presence of myelin specific autoantibodies in the regions of demyelination plaques in patients with MS (7). In addition, the identification and quantitation of myelin basic protein (MBP)- and myelin oligodendrocyte glycoprotein (MOG)-targeted autoantibodies to MBP and MOG have proven useful for diagnostic and prognostic purposes (8–10). Nonetheless, the mechanisms responsible for the induction of autoantibodies and their possible contributions to MS progression are still unknown and are somewhat controversial (11). Over the last two decades, the use of different modes of magnetic resonance imaging (MRI) (12, 13) has proven to be very important for obtaining objective evidence for the dissemination of MS in time and space. The use of other biomarkers has been shown to provide important information for the verification of diagnosis and the establishment of prognostic criteria and for evaluating the efficacy of therapeutic interventions (14, 15). The identification of new MS biomarkers could thus be of importance for diagnostic, prognostic, and therapeutic purposes (16).

During the last 10 years, it has been found that Abs contribute to the degradation of a number of autoantigens. These and related “antibody-enzymes”, also termed azymes, were shown to be able to cleave DNA, RNA, carbohydrates, peptides, and proteins (17). Specific catalytic B cell responses were associated with cancers as well as with autoimmune, immune, infectious, and inflammatory disorders including asthma, Hashimoto’s thyroiditis, autoimmune myocarditis, systemic lupus erythematosus, scleroderma, rheumatoid
Figure 1. Interaction of human Abs with MBP, MOG, and MBP recombinant fragments. A, Amino acid sequence of MBP in relation to areas of Ab binding. Serum was obtained from MS patients at different stages of RR and PP/SP disease, as well as patients with OND and healthy donors (HD). Regions that distinguish the binding features of Abs from two groups are shown in color. High-affinity fragments are shown as dark blue, low-affinity fragments as light blue, and nonbinders as white. B, Box plots represent the epitope specificity of autoantibodies toward MBP recombinant fragments as well as intact MBP and MOG proteins as measured by ELISA. Numbers above the panels indicate the inclusive amino acids of each fragment of MBP; Trx indicates thioredoxin protein carrier alone. For group comparisons, significance values from *p < 0.05 are indicated; NS, p > 0.05. The median in each group is shown by the bold line, the box plots represent the second and the third quartiles of the distribution, and the error bars represent the 95% confidence intervals. Numbers on the vertical axes indicate OD₄₅₀.

### Materials and Methods

**Patients and healthy donors**

Frozen serum samples were obtained from the Vladimirsky Moscow Regional Clinical Institute and Clinical Hospital of the Russian Academy of Sciences (Moscow, Russia). Autoantibody purification and characterization were done with sera from 26 MS patients, 22 other neuronal disorder (OND) patients who had not been treated with steroids or nonsteroidal anti-inflammatory drugs, and 11 healthy donors. The MS patients were between 16 and 72 years of age (median 39.5 years; interquartile range from 28 to 48 years). Their scores on the expanded disability status scale (EDSS) ranged from 0 to 6.5 (median 3.2 ± 1.8). The EDSS is scored on...
a scale of 0 to 10, with higher scores indicating greater disability. The EDSS values were calculated according to the Poser classification of disease progression that combines clinical, immunological, and MRI data analysis (30). Cohorts of the OND patients and healthy donors were a median 65 years (interquartile range from 39 to 72 years) and 33.5 years (interquartile range from 26 to 47 years) of age, respectively. Informed consent was obtained as approved by the Institutional Review Board of the Vladimirsky Moscow Region Clinical Institute and Clinical Hospital of the Russian Academy of Sciences in accordance with the regulations of the Ministry of Health of the Russian Federation (Moscow, Russia).

**Purification of IgG**

IgGs from sera of humans were purified essentially as described (24). IgG was isolated from serum by thrice-repeated 50% ammonium sulfamate precipitation followed by affinity chromatography on protein G-Sepharose (Amersham Biosciences). IgG-containing fractions were then dialyzed against PBS or TBS with 0.05% NaN3 at 4°C. The IgG amount was quantified and standardized by ELISA. IgG purity was assessed by electrophoresis followed by silver staining, immunoblotting under nonreducing conditions and by surface-enhanced laser desorption/ionization (SELDI) mass spectrometry.

**Synthesis, cloning, expression, and purification of the extracellular domain of MOG and thioredoxin (Trx)-fused MBP peptides (MBP epitope library)**

A DNA fragment corresponding to the extracellular Ig-like domain of MOG was amplified by PCR with the specific primers 5′-GAGGAAGGC ATGGCGAGGGCAGTTCAGGATA3′- and 5′-AGAGAGGAGAT CTCTCCTTTCAATCCATTCCG-3′ using human genomic DNA as the initial template. To obtain pET32CH, sequence coding the c-myc epitope was added to the pET32b+ plasmid (Novagen) using BamHI and EcoRI restriction sites for the unambiguous identification of recombinant proteins by a monoclonal anti-c-myc Ab. The NcoI-BglII PCR product was cloned into pET32CH using NcoI and BamHI restriction sites. Twelve DNA fragments encoding human MBP peptides, 1–27, 17–41, 25–54, 43–68, 53–81, 65–92, 81–103, 81–103 (substitution R97A), 91–114, 107–132, 123–140, 130–156, and 146–170, were prepared by PCR with four overlapping oligonucleotides for each of them and were ligated to a DNA fragment corresponding to the linker (SGGGG)-S. The final PCR products were cloned in-frame into the pET32CH plasmid using NcoI and BamHI restriction sites. The expression products of plasmids that contained N-terminal Trx fused through the (SGGGG)-S linker to the MBP peptides (MBP epitope library) were used for cleavage and binding analysis. The plasmid encoding Trx with linker (SGGGG)-S was designed as a control. The soluble recombinant His-tagged proteins were obtained by Escherichia coli expression and isolated by sorption on a Talon SuperFlow (BD Biosciences) column followed by cation exchange chromatography on a Mono S column (Amersham Biosciences) at pH 5.0 and subsequent size exclusion chromatography on a Superdex 75 GL 10/300 column (Amersham Biosciences) in 150 mM NH4HCO3 buffer.

**MBP preparations**

MBP was prepared from bovine brain according to Ref. 31; the obtained protein was purified by reverse phase HPLC on column C4 10/250 (Mashery-Nagel).

**Synthesis, cloning, expression, and purification of encephalitogenic peptide fluorescent resonance energy transfer (EPeFRET)**

A pQe30-based vector (Qiagen) encoding the PS-CFP2 and TurboYFP proteins (both from Evrogen) interconnected with the short peptide linker representing MBP fragment 81–103 (EPeFRET) was done by replacement of the sequence, encoding the initial peptide linker in a single cloning step with the use of two oligonucleotides. Fusion proteins were expressed in the BL21 E. coli cells. After the OD600 of the cell culture reached 0.6–0.8 U at 37°C, it was incubated at room temperature for 24–36 h for chromophore maturation. The soluble recombinant His-tagged proteins were further isolated by sorption on a Talon SuperFlow (BD Biosciences) column followed by size exclusion chromatography on a Superdex 75 GL 10/300 column (Amersham Biosciences) in PBS or TBS. Purified proteins were incubated at 10°C for 1 wk for complete fluorescent protein chromophore maturation in PBS/TBS buffer with 0.05% NaN3. The fluorescence spectra of fluorescent resonance energy transfer (FRET) fusion proteins were obtained using a Cary Eclipse fluorescence spectrophotometer (Varian). The excitation wavelength was 405 nm (the maximum absorption

**FIGURE 2.** Correlation of the EDSS of MS patients with the levels of autoantibodies to MBP fragments. Symbols indicate OD450 values for Abs from individual subjects within each EDSS category for specific MBP peptides or intact MBP and MOG proteins. Lines indicate the relation between EDSS value and the binding activity of specific peptides/proteins. MBP 81–103 (R2 = 0.332; p = 0.024), MBP 130–156 (R2 = 0.381; p = 0.011), MBP 146–170 (R2 = 0.310; p = 0.033), intact MBP (R2 = 0.458; p = 0.005), intact MOG (R2 = 0.520; p < 0.001), and control Trx carrier protein (R2 = 0.055; p = 0.376).

for PS-CFP2 protein) and the emission maximum was observed on 538 nm (corresponding to the TurboYFP (Evrogen) emission maximum).

**Proteolysis of MBP epitope library by abzymes and enzymes**

Purified Abs (0.1–2 μg) were incubated at 37°C for 14 h in the final volume of 20 μl PBS and 0.02% NaN3 containing 2 μg of recombinant MBP peptides. Matrix metalloprotease 3 (MMP-3; Sigma-Aldrich) and cathepsin D (Sigma-Aldrich) were incubated with 2 μg of MBP fragments at 37°C for 3 h in an enzyme-substrate molar ratio of 1:100 in final volume of 20 μl of 50 mM Tris (pH 7.5), 5 mM CaCl2, and 50 mM NH4OAc (pH 3.5), respectively. Bovine trypsin (Sigma-Aldrich) was incubated with 2 μg of MBP peptides for 2 h at 37°C in an enzyme-substrate molar ratio of 1:200 in final volume of 20 μl of 50 mM Tris (pH 7.5), and 5 mM CaCl2. The reaction was stopped by the addition of an equal volume of Laemmli’s buffer. The extent of fusion protein degradation was visualized by SDS-PAGE in a Tris-glycine buffer system.

**Kinetic measurements of EPeFRET hydrolysis by abzymes and enzymes**

Kinetic measurements were performed using different EPeFRET concentrations (0.25–30 μM) in PBS for abzymes or TBS and 5 mM CaCl2 for an enzyme investigation. The acetic buffer at pH 3.5 requested for cathepsin D was not applicable due to the great decrease of FRET at such a pH. Th abzymes’ final concentration was 80 nM, and bovine trypsin and MMP-3 were 40 and 30 nM, respectively. Cathepsin D was inactive in the same concentration range. Inhibition was done using a constant 1 μM EPeFRET concentration and various concentrations of 0.001–100 μM Trx-presented MBP 81–103, its mutant R97A, the Trx carrier, and Cопаксон. Abzymes’ final concentration was 80 nM and bovine trypsin and MMP-3 were at 10 nM. The reaction was conducted in a volume of 120 μl in 96-well black plates (Greiner) and measured by a Genius microplate reader (Tecan) using a 405-nm excitation filter and a 535-nm emission filter. Reaction velocities and IC50 values were calculated using the linear and sigmoidal regression engines, respectively, of the SigmaPlot 2001 enzyme kinetics 1.10 module (SPSS).

**SELDI analysis of EPeFRET hydrolysis by Abs and enzymes**

SELDI analysis of Trx fusion peptide cleavage was performed using H4 ProteinChips and ProteinChip Reader PBS IIC (Ciphergen Biosystems), according to the manufacturer’s protocol.
Microtiter plates (Maxisorp Nunc) were coated with 50 μL of a 10 mg/L solution of recombinant MBP peptides, MBP, and MOG, in 100 mM carbonate/bicarbonate buffer (pH 9.6) in odd column wells. All even column wells were coated with 100 mM carbonate/bicarbonate buffer (pH 9.6). Plates were sealed with ELISA plate sealer (Costar) and incubated at 4°C overnight and then washed (300 μL/well) three times with PBS containing 0.15% Tween 20. All wells were then blocked with 300 μL of 2% BSA (Sigma-Aldrich) in carbonate/bicarbonate buffer (pH 9.6) and incubated for 1 h at 37°C. The plates were washed with PBS containing 0.15% Tween 20. Purified Abs were preincubated with Trx immobilized on Sepharose 4FF and diluted in PBS containing 0.15% Tween 20, 5 United States Pharmacopeia (USP) units of heparin per milliliter (in case of MBP as an Ag), and 0.5% BSA at the final dilution of 1/60 to 1/500, and 50 μL of the diluted

**FIGURE 3.** Proteolytic targets in MBP (A) and its epitope library (B–G). A, Previously reported sites of MBP proteolysis by different proteases (33, 35, 37, 46, 48), abzymes (24), and autocatalytic hydrolysis (34) (top section) and MBP sequence with marked cleavage sites (bottom section). The sequence of the encephalitogenic peptide 81–103 is shown in red. B, Absence of hydrolysis of the epitope library by Abs from healthy donors (n = 11/11), OND (n = 20/22), and MS patients (n = 5/26). C and D, Clearly established hydrolysis of MBP fragments by abzymes from one representative MS patient (n = 21/25) (C) and by IgG from one of the two OND patients that demonstrated cleavage activity (n = 2/22) (D). E–G, Degradation of MBP peptides by purified bovine trypsin (E), cathepsin D (F), and MMP-3 (G). Purified Abs (0.1–2 μg) were incubated with 2 μg of recombinant Trx-presented MBP peptides for 14 h at 37°C. MMP-3 and cathepsin D were incubated with 2 μg of MBP peptides for 3 h at 37°C in an enzyme-substrate molar ratio of 1:100. Bovine trypsin was incubated with 2 μg of MBP peptides for 2 h at 37°C in an enzyme-substrate molar ratio of 1:200. The reaction was stopped by the addition of an equal volume of Laemmli’s buffer. The extent of fusion protein degradation was visualized by SDS-PAGE in a Tris-glycine buffer system. The control Trx carrier is in the far left lanes (B–G) and the MBP peptides in the other lanes are listed at the top. The cleavage fragments from peptides 81–103 and 91–114 are indicated by arrowheads.
sample was added to each well of the plate. mAbs to MBP (MAB381; Chemicon) and MOG (clone 8.18-C5; M. Reindl, University of Innsbruck, Austria) and known positive and negative human serum samples were used as controls. The plates were incubated for 1 h at 37°C and washed three times with PBS and 0.15% Tween 20. To each well, 50 μl of tetramethyl benzidine (US Biological) was added to each well and stored in the dark from 5 to 15 min. The reaction was stopped with 10% phosphoric acid (Merck) at 50°C. The OD450 values were measured by a Genius microplate reader (Tecan).

Statistical analysis

Statistical analysis was performed with SPSS 13 software using a non-parametric Mann-Whitney U test and Spearman rank correlation. The results are expressed as medians and interquartile ranges. All reported p values are two-sided; p < 0.05 was considered indicative of statistical significance.

Results

Analysis of binding activity of autoantibodies to MBP epitope library

Serum samples from 26 patients with MS, 22 patients with OND, and 11 healthy individuals were analyzed by ELISA for the levels of Abs to intact MBP and MOG proteins and the MBP epitope library (Fig. 1). A comparative analysis of the level of autoantibodies toward two major neuronal Ags and the MBP peptides presented in solution may shed light on the existing controversial results concerning diagnostics for the progression

### Table 1. Sites of hydrolysis of MBP peptide 81–103 from epitope library by autoantibodies from MS and OND patients, bovine trypsin, cathepsin D, and MMP-3

| Catalysta | MS Stagec | 81QDENPVVH | F | F | K | N | I | V | T | P | R | T | PPPSQ103 |
|-----------|-----------|-------------|---|---|---|---|---|---|---|---|---|---|---|---------|
| MS32      | RR/R      | X X         | X | X |   |   |   |   |   |   |   |   |         |
| MS37      | RR/R      | X X         |   |   |   |   |   |   |   |   |   |   |         |
| MS38      | RR/E      | X X         | X | X |   |   |   |   |   |   |   |   |         |
| MS39      | RR/E      | X X         |   |   |   |   |   |   |   |   |   |   |         |
| MS40      | RR/R      | X X         |   |   |   |   |   |   |   |   |   |   |         |
| MS41      | RR/R      | X X         |   |   |   |   |   |   |   |   |   |   |         |
| MS42      | RR/R      |   X X       |   |   |   |   |   |   |   |   |   |   |         |
| MS43      | RR/R      |   X X       |   |   |   |   |   |   |   |   |   |   |         |
| MS44      | SP/E      |   X X       |   |   |   |   |   |   |   |   |   |   |         |
| MS45      | SP/E      |   X X       |   |   |   |   |   |   |   |   |   |   |         |
| MS46      | SP/R      |   X X       |   |   |   |   |   |   |   |   |   |   |         |
| MS47      | SP        |   X X       |   |   |   |   |   |   |   |   |   |   |         |
| MS48      | SP        |   X X       |   |   |   |   |   |   |   |   |   |   |         |
| MS49      | SP/E      |   X X       |   |   |   |   |   |   |   |   |   |   |         |
| MS50      | SP/E      |   X X       |   |   |   |   |   |   |   |   |   |   |         |
| Trypsinf  |          | X X         |   |   |   |   |   |   |   |   |   |   |         |
| MMP-3     |          | X X         |   |   |   |   |   |   |   |   |   |   |         |
| Cathepsin Dg |          | X X         |   |   |   |   |   |   |   |   |   |   |         |

a Purified Abs (0.1–1 μg), MMP-3, cathepsin D, and bovine trypsin were incubated with 1 μg of recombinant MBP peptide 81–103 from epitope library. MMP-3 and cathepsin D were used an enzyme-substrate molar ratio of 1:100 and bovine trypsin at a molar ratio 1:200. The reaction was stopped by the addition of 10 volumes of 50% acetonitrile plus 0.1% trifluoroacetic acid, and 1 μl from this mixture was subjected to SELDI mass spectrometry. Major sites are marked by a boldfaced X and minor sites by a lowercase x.

b The MS designations refer to IgG purified from the MS patients with respective numbers. Abs from two of 22 patients with OND were positive as well. Cleavage sites were minor and located after arginine 97 (data not shown).

c E, Exacerbation; R, remission.

### FIGURE 4.
A, Principal scheme of analysis by FRET technique of enzyme and abzyme-mediated cleavage of encephalitogenic peptide. B, Hydrolysis of EPeFRET containing the MBP encephalitogenic fragment 81–103 by abzymes (kcat/Km, 1.8 × 10^4 s⁻¹ M⁻¹) compared with the control sample. C, Protodelysis of EPeFRET by MMP-3 (kcat/Km, 3.8 × 10^4 s⁻¹ M⁻¹) and bovine trypsin (kcat/Km, 2.5 × 10^4 s⁻¹ M⁻¹) compared with the control sample. Figures showing the fluorescence spectra of EPeFRET cleavage reactions by abzymes and bovine trypsin are given in the B and C insets. Kinetic measurements were performed using different EPeFRET concentrations (0.25–30 μM) in PBS for abzymes and TBS and 5 mM CaCl₂ for investigation of enzymes. Final concentration of the abzymes was 80 nM and those of bovine trypsin and MMP-3 were 40 and 30 nM, respectively. Cathepsin D was inactive in the same concentration range. D–G, Hydrolysis of EPeFRET by abzymes, bovine trypsin, and MMP-3 is inhibited by MBP peptide 81–103 (D), mutant MBP peptide 81–103 (R97A) (E), Copaxone (F), and Trx carrier (G). Iₐₜ values for abzyme activity are shown in D–G. Inhibition was done using constant 1 mM EPeFRET concentration and various concentrations (0.001–100 μM) of the MBP peptide from epitope library 81–103, its mutant R97A, Ox carrier, and Copaxone. Final concentration of the abzymes was 80 nM and those of bovine trypsin and MMP-3 were 10 nM. All measurements were performed in three independent assays. AU, Arbitrary unit.
of MS and the conversion of the clinically isolated syndrome (CIS) to clinically definite multiple sclerosis (CDMS) (8–11). We hypothesized that these contradictory outcomes are the consequence of using whole MBP and MOG molecules as Ags, where the intrinsic role of the concrete functional epitope is masked. We constructed a recombinant library of the overlapping peptide fragments covering the entire MBP molecule fused in-frame with Trx (MBP epitope library). The carrier protein mediates “presentation” of these epitope fragments to the IgG molecule in solution, i.e., leads to their better recognition and interaction with Abs. Such methodology helps to avoid possible difficulties with the expression, instability, and solubility of Ags as well. The levels of autoantibodies to most of the MBP fragments generated from aa 43 to 170 as well as intact MBP and whole MOG molecules were significantly higher in sera from MS patients than in sera from healthy donors (Fig. 1A, lines 3 and 4). In contrast, sera from MS and OND patients were distinguished only by the levels of Abs to MBP fragments 48–60 and 146–170 (Fig. 1, lines 1 and 2). The analysis of other neurological and connective tissue diseases different from MS was due to the fact of MS mimicking by those disorders (32). Interestingly, that epitope spreading may be clearly observed while comparing relapsing-remitting and progressive MS (Fig. 1, lines 3 and 4). The EDSS of MS patients correlated with the levels of autoantibodies to MBP fragments 81–103 ($R^2 = 0.332; p = 0.024$), $130–156 (R^2 = 0.381; p = 0.011), 146–170 (R^2 = 0.310; p = 0.033), intact MBP (R^2 = 0.458; p = 0.005),$ and whole MOG proteins ($R^2 = 0.520; p < 0.001$) but not to reactivity with the control Trx carrier protein ($R^2 = 0.055; p = 0.376$) (Fig. 2). Thus, using the MBP epitope library instead of the whole molecule allows the design of a new possible biomarker for MS. Moreover, all these data show a more effective and precise method to distinguish MS and OND, keeping in mind the acuteness of this problem.

Analysis of cleavage activity of autoantibodies to MBP epitope library

Fig. 3A represents an evaluation of the sites of whole MBP molecule hydrolysis by a set of natural enzymes and catalytic Abs (abzymes). Both types of catalysts are thought to be involved in MS pathogenesis to a variable degree (25, 29, 33–37). Locations of the sites are quite near to one another, but the cleavage pattern is unique in each case. The major activity is concentrated around the MBP peptide 81–103, emphasizing its immunodominant properties. In this study we analyzed the peculiarities of substrate properties of entire MBP molecule vs its epitope library in enzyme and abzyme-mediated cleavage reaction. IgGs purified from sera of the same sets of patients and controls were analyzed for specificity of MBP-derived recombinant epitope library cleavage (abzyme activity). The results were compared with the patterns of degradation generated by purified bovine trypsin, cathepsin D, and MMP-3. As shown in Fig. 3, C and D, the abzymes hydrolyzed only the MBP peptides 81–103 and 91–114, which are close to MMP-3 (65–92 and 81–103; Fig. 3G). In contrast, bovine trypsin and cathepsin D were active to some extent against all fragments (Fig. 3, E and F). Abzyme-mediated cleavage of the two MBP peptides was primarily after arginine 97 and, to a lesser extent, after lysine 91, while MMP-3 hydrolyzed the respective peptides after phenylalanine 89 (F89; see Table I). Bovine trypsin also hydrolyzed encephalitogenic peptide after lysine 91 and arginine 97 but with reversed site preference and an additionally cleaved Trx carrier. Cathepsin D hydrolyzed only the carrier protein and had no sites inside the MBP fragments. As a specificity control, we tested the Abs against a mutant MBP 81–103 peptide in which arginine 97 was replaced by an alanine; this peptide was susceptible to MMP-3 but not to Ab-mediated catalysis (data not shown). As shown in Table I, Abs from 11 of 13 (85%) relapsing-remitting (RR) and 10 of 13 (77%) primary and secondary progressive (PP/SP) MS patients were positive for cleavage. Three and two of 13 IgG samples, respectively, were lysine 91-arginine 97 double positive. Interestingly, among neither RR-MS nor PP/SP-MS cohorts could we find patients with IgG proteolytic activity against single lysine 91. In comparison, Abs from none of the 11 healthy controls and from only two of 22 patients (9%) with OND were positive. Cleavage sites were minor and were located after arginine 97. Based on these data, we present an additional possibility to differential MS diagnostics by using abzymatic activity toward MBP epitope library.

Analysis of cleavage activity of autoantibodies and enzymes to encephalitogenic MBP peptide using FRET technique

To enable high throughput analyses of peptide cleavage, we engineered a FRET construct, designated EPeFRET, with the MBP encephalitogenic peptide 81–103 flanked by two fluorescent proteins, PS-CFP2 and TurboYFP (both from Evrogen). By design, energy transfer between the two chromophores should be interrupted by cleavage of the peptide (Fig. 4A). Studies using purified catalytic IgG from the serum of a representative MS patient as well as model proteases (bovine trypsin, cathepsin D, and MMP-3) showed that the peptide retained its established substrate properties (Table II and Fig. 4, B and C). The catalytic constant for abzymes (0.014 s$^{-1}$) was three orders lower than that for bovine trypsin (8.8 s$^{-1}$). However, due to a better Michaelis constant the catalytic efficiency ($k_{cat}/K_m$) lost only two orders in comparison with bovine trypsin (1.8 × 10$^3$ and 2.5 × 10$^5$ s$^{-1}$ M$^{-1}$, respectively). Catalytic efficiency of the MMP-3 (3.8 × 10$^4$ s$^{-1}$ M$^{-1}$) was in the middle range of the
obtained activities. In addition, as shown in Table II and Fig. 4, D–G, abzymatic hydrolysis of the EPeFRET in the concentration of 1 μM was efficiently inhibited by the MBP peptide 81–103 from the epitope library (IC50 = 0.007 μM) and less well by its mutant 81–103 (R97A) (IC50 = 0.09 μM) or glatiramer acetate (Copaxone) (IC50 = 0.1 μM); the carrier protein did not have any effect on catalysis (IC50 >> 10 μM). In contrast, these substances had little if any effect on the cleavage of the FRET peptide by bovine trypsin or MMP-3. IC50 values for MMP-3 were similar for all tested inhibitors and were contained in the interval from 8.2 to 20.0 μM (Fig. 4, D–G, and Table II). Bovine trypsin was sufficiently inhibited only by Copaxone (Fig. 4F and Table II), due to, it seems, its chemical structure, which contains ~29% of lysine residues.

Discussion
The prospects for progress in developing novel approaches for the diagnosis and treatment of MS have been greatly encouraged by several observations. These include: 1) the identification of a major immunogenic region in MBP, peptide 82–98; 2) the demonstration that both B and T cell responses to MBP are focused on this region, particular for patients who express HLA-DR2; and 3) clinical studies suggesting that patients treated with MBP peptide 82–98 can be made tolerant to the protein in association with delayed disease progression or reduced disease activity over time (1). The studies described here suggest important opportunities for improving diagnostic, prognostic, and therapeutic approaches to MS. This view is based on the demonstration that serum Abs from patients with MS exhibit abzyme activity toward MBP and that a novel FRET-based assay for MBP peptide cleavage can be used to screen for this activity. These findings derive from the use of MBP peptides rather than intact MBP or MOG proteins to assay for Ab binding and cleavage. We have described two distinct methods for detecting anti-myelin Abs: binding and catalysis.

Binding
The use of assays for Abs to MBP and MOG for diagnostic and prognostic purposes in patients with a CIS, a frequent precursor to CDMS, has yielded conflicting results. One study showed that the presence of myelin Abs in the sera of CIS patients was predictive of a shorter time course to the development of CDMS (8). Others, however, indicated that positive tests had no prognostic value for progression to CDMS (11). The latter results are in keeping with our demonstration that the binding activities of Abs to intact MBP and MOG proteins were indistinguishable for patients with MS and OND. In addition, a screen of MBP peptides showed that the reactivity of Abs from patients with MS and OND could be distinguished only by their differential binding to MBP 43–68 and MBP 146–170. Follow-up studies of patients with MS using assays based on the use of these fragments will be needed to determine whether it provides an improved prognostic tool for patients with CIS.

Interestingly, the parameters of Ab binding associated with disease progression were different from those with potential prognostic value. Disease severity, as determined by EDSS, correlated with level of autoantibodies to intact MBP and MOG proteins as well as MBP fragments 81–103, 130–156 and 146–170. These findings are in keeping with the demonstrated correlations between levels of anti-MOG and anti-MBP Abs and inflammatory signs revealed by MRI and cerebrospinal fluid analyses (9).

Catalysis
The neurodegenerative model of MS is based on the proposition of a primary lesion in myelin followed by myelin breakdown and the release of myelin-compartmentalized proteins, particularly MBP. This is followed by the generation of MBP-derived peptides that become the main sensitizers of T cells (1). The hypothesis that interactions between MBP and T cells must occur at sites other than the myelin membrane has been challenged by recent physicochemical studies demonstrating that myelin can become structurally unstable secondary to specific posttranslational modifications of MBP structure. This was shown to result in the increased surface exposure and susceptibility to proteolysis of MBP 83–92 (29, 36). The close association between the proteolytic activity of MBP and the pattern of posttranslational modifications of the molecule may represent one of the key regulatory mechanisms in epitope generation (38, 39). Proteolysis may proceed by any of four distinct pathways that may exert a concerted attack on the MBP molecule, although the mechanisms responsible for the activation and regulation of these potential activities are not known. Thus, epitope generation may occur via: 1) autocatalytic cleavage of the MBP molecule (34); 2) protease digestion (33, 40); 3) autoantibody-mediated site-specific cleavage (24); or 4), an Abdependent oxidative pathway (41). The last mechanism, which has been demonstrated in a number of autoimmune pathologies, remains unproven for MS (42). These pathways are characterized by dramatic differences in reaction velocities and in cleavage site specificity. Obviously the rates of enzymatic reactions are several times higher than those for autocalysis and Ab enzymes (abzymes) (24, 33, 34), perhaps making this pathway the major player in epitope generation. In the case of abzymes, the large excess of the biocatalyst, its high specificity, and its close compartmentalization with the MBP substrate shown in demyelinating lesions (7) are suggestive of its likely effectiveness in vivo. This was documented for the cleavage of Bence-Jones proteins in multiple myeloma and the Ab-mediated cleavage of factor VIII in hemophilia and in sepsis (21, 43, 44). As shown previously, the velocity of the MBP-specific abzyme reaction is at the upper limit of published disease-associated Ab catalysts (24). The conformational instability and flexibility of the MBP molecule together with a rather wide spectrum of posttranslational modification makes MBP with its various isoforms a most unusual substrate. Pathways 1 (autocatalytic cleavage of the MBP molecule) and 3 (autoantibody-mediated site-specific cleavage) may be combined and, to some extent, abzymes may play the role of effective “templates” for autocatalytic processing as suggested earlier for DNA abzymes in systemic lupus erythematosus (19). It has been suggested that the normal proteolytic processing of MBP destroys the encephalitogenic epitope, thus bypassing central tolerance and predisposing to autoimmune attack (45). A systematic evaluation of enzyme and abzyme cleavage sites within MBP showed that some of the abzyme targets were identical with the sites cleaved by one of the enzymes (Fig. 3A). Other abzyme cleavage sites were unique but often displaced by only one or a few amino acids from those targeted by one or more enzymes. As mentioned earlier, the site specificity of enzyme cleavage is greatly influenced by posttranslational modifications and the specific isoforms of MBP (33, 46). By comparison, abzyme-mediated cleavage of MBP is unaffected by phosphorylation status, indicating that this biocatalyst is less regulated by the physiological alterations of target proteins (24). This feature of
abzyme active sites may ensure consistency in MBP-derived epitope processing.

As we demonstrated previously (24), catalytic Abs to the encephalitogenic peptide are present in the serum of the MS patients. In this study we significantly expanded the number of MS patients studied for them specificity of MBP peptide cleavage as well as examining patients with a broader range of OND. We showed that sera from normal controls do not exhibit MS-defined cleavage activity. The rigorous purification scheme first developed for the isolation of “natural abzymes” (47) ensures that the activity measured is abzyme-mediated and cannot be ascribed to contaminating enzymes. Among the OND patients who were studied we could find only 9% positives cases of abzyme catalysis. These findings indicate that site-specific, abzyme-mediated cleavage of defined MBP peptides may represent a novel diagnostic method for MS, with the level of activity providing an additional biomarker of disease progression.

The use of encephalitogenic peptides from an epitope library fused with fluorescent proteins provides a novel FRET approach to screen for the presence and activity of catalytic autoantibodies.

The encephalitogenic peptide and its mutant as well as Copaxone were shown to be good inhibitors of abzyme-mediated FRET hydrolysis. The therapeutic benefit of Copaxone and encephalitogenic peptide for patients with MS is consistent with the model showing that catalytic Abs may play some role in the pathogenesis of this disease (4, 45).

The data presented here and previously (24, 25) suggest that abzyme-mediated cleavage of MBP peptides may provide a novel biomarker to complement established magnetic resonance imaging approaches to the diagnosis and staging of MS. Further studies of the features of MBP-specific abzymes may open new perspectives in the development of MS therapeutics while clarifying the mechanisms of action of existing drugs.

Disclosures
The authors have no financial conflict of interest.

References
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