Autocatalytic Cleavage of Myelin Basic Protein: An Alternative to Molecular Mimicry†

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ABSTRACT: Although multiple sclerosis (MS) is thought to be an autoimmune disease, the mechanisms by which immunodominant epitopes are generated and lymphocytes are activated are not known. Here, myelin basic protein−component 1 (MBP-C1) from MS tissue was shown to undergo autocatalytic cleavage at slightly alkaline pH. Importantly, one of the major peptides released contained the immunodominant epitope 84−89. Interestingly, MBP isolated from MS patients showed a faster time course of cleavage and a more robust release of epitope 84−89 than MBP isolated from normal individuals. The cleavage reaction was not inhibited by protease inhibitors, except for phenylmethanesulfonyl fluoride (PMSF), a serine protease inhibitor. Since PMSF inhibition suggested a role for a serine residue in the cleavage, we labeled myelin basic protein with diisopropyl fluorophosphate (DFP), known to bind active site serine residues. Mass spectrometry was used to identify the labeled peptide, which consisted of residues 140−152. Since this peptide contained a single serine residue, we concluded it to be the active serine. The importance of this cleavage mechanism is that it provides for a ready source of the immunodominant peptide for sensitization of T-cells. It is not necessary to invoke other mechanisms such as molecular mimicry.

Multiple sclerosis (MS),[1] an inflammatory demyelinating disease of the human central nervous system, is characterized by multiple lesions within the white matter (*1*, *2*). MS is a disease heterogeneous in its pathophysiology and clinical manifestations. Reflecting this heterogeneity, a combination of factors is thought to be involved in its etiology, including genetic, environmental, and immunological, although its exact etiology remains unknown. MS has traditionally been viewed as an autoimmune disease, i.e., a disease resulting from an attack of the immune system on self-proteins. While the immune system plays a role in the pathogenesis and progression of the disease, an autoimmune attack as an initiating event has not been shown.

The problem with the autoimmune theory is explaining how sensitization of T-cells in the periphery can take place before myelin breakdown. Molecular mimicry was considered to provide the answer (*3*). In this theory, cross-reactivity between proteins of different genetic background is considered responsible. Thus, microbial peptides, especially viral proteins with sequences similar to those of one of the myelin proteins, sensitize T-cells in the periphery which then recognize a myelin antigen and destroy myelin. However, more recently, the mimicry has been shown to be more sophisticated than amino acid sequence alone. Conformational similarities allowing a peptide of different amino acid sequence to fit into the MHC pocket may also sensitize T-cells (*4*−*6*).

Despite its attractiveness as a theory, molecular mimicry has never been shown to be a factor in any disease (*5*) including multiple sclerosis. Another mechanism receiving more prominence recently is the neurodegenerative hypothesis which postulates a lesion in myelin, possibly altering compaction, occurs first, followed by myelin breakdown and release of myelin proteins, in particular, myelin basic protein (MBP). Peptides of MBP sensitize T-cells in the periphery which then travel to and destroy myelin.

In the human, MBP is an 18.5 kDa protein and is a family of similar proteins generated by various posttranslational modifications. Detailed studies have shown that it contains two immunodominant epitopes. The first and major epitope is in the region of residues 84−102 (*7*). The second epitope is in the C-terminus of the molecule in the area of residues 139−153 (*8*). In MS, the immunodominant epitope has been localized to 70−89 and 83−97 (*9*, *10*). Therefore, this area of the molecule has special significance for disease.

Proteolytic cleavage of MBP is generally thought to be the mechanism for the generation of epitopes. We report here a novel mechanism. We have found that autocatalysis (the cleavage of peptide bonds without the addition of other factors) of MBP from MS tissue generates a peptide containing the immunodominant epitope. MBP from normal

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[1] Abbreviations: MS, multiple sclerosis; MHC, major histocompatibility complex; MBP, myelin basic protein; CM52, carboxymethylcellulose; MS C1, multiple sclerosis component 1; N C1, normal component 1; MALDI-QTOF, matrix-assisted laser desorption/ionization−quadrupole time of flight; DFP, diisopropyl fluorophosphate; HPLC, high-performance liquid chromatography; BSA, bovine serum albumin; TCA, trichloroacetic acid; mAb22, monoclonal antibody 22; PMSF, phenylmethanesulfonyl fluoride; NAWM, normal appearing white matter.

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tissue carries out this reaction only at very minimal rates. Therefore, autocatalysis of MBP can produce the immunodominant peptide which can sensitize T-cells. We report on the properties of this reaction and show that a single serine (residue 151) out of a total of 18 seryl residues in MBP is involved in the catalysis.

MATERIALS AND METHODS

Isolation and Purification of MBP-C1 from Normal and MS Brain. Human white matter was homogenized in 2:1 (v/v) chloroform−methanol and stirred overnight at 4 °C. The homogenate was filtered through Whatman no. 1 filter paper, and the residue was washed with chloroform−methanol followed by cold acetone. The residue was then homogenized in 0.2 N H_2SO_4 and stirred overnight at 4 °C. The homogenates were centrifuged at 11000*g* for 60 min at 4 °C, and the supernatant was decanted. The protein was precipitated in absolute ethanol (−10 °C) overnight and was centrifuged at 11000*g* for 60 min. The pellets were washed three times in cold ethanol, resuspended in buffer (6 M urea, 80 mM glycine, pH 9.5), and dialyzed overnight.

MBP exists as a family of charge isomers which differ in net charge and result from various posttranslational modifications. To isolate individual charge isomers of MBP, the isolated MBP was loaded onto a CM52 cation-exchange column, and the components were eluted with a 0−0.2 M NaCl gradient in 0.08 M glycine buffer (pH 10.5) containing 2 M urea (*11*). The fractions collected were monitored at 280 nm. Component 8 was found in the void volume while the more cationic components (C5, C4, C3, C2, and C1) eluted with an increasing salt gradient. The components were dialyzed against water, lyophilized, and stored at −20 °C.

Autocleavage of C1 and Western Blotting. MBP-C1 isolated from MS patients (MS C1) or from normal individuals (N C1) was incubated in 50 mM Tris, pH 7.5, 5 mM $CaCl_2$, and 0.02% NaN_3 at 37 °C. At various time intervals, aliquots (2 μg) were removed, added directly to sample buffer, and frozen. Aliquots were run on 10−20% gradient gels (Invitrogen, Burlington, Canada), and Western blots were done using either a monoclonal antibody to MBP 84−89 (mAb22; Serotec, Raleigh, NC) or a polyclonal MBP antibody (gift from Dr. Eugene Day, NC). Experiments run under various conditions of pH and temperature were carried out similarly. Buffers used for the different pH conditions were 50 mM glycine hydrochloride buffer, pH 2.0, 50 mM sodium acetate buffer, pH 4.0, 50 mM sodium acetate buffer, pH 6.0, 50 mM Tris buffer, pH 7.5, and 50 mM Tris buffer, pH 8.5.

Elution and Identification of Peptides from Gels. Peptides resulting from the autocleavage of C1 were eluted from gels using method A of Cohen and Chait (*12*) for further analysis by mass spectrometry or Edman degradation. Briefly, gels were stained in Coomassie Blue made up in 50% methanol and subsequently destained in 50% methanol. Stained peptide bands were extracted using a scalpel and placed into separate Eppendorf tubes. Bands were destained completely in 30% acetonitrile and 70% 100 mM ammonium bicarbonate. Bands were crushed and extracted with formic acid−water−2-propanol at room temperature for 2 h. The supernatant was collected and lyophilized until ready for use. Peptide masses were obtained by mass spectrometry on a MALDI-QTOF mass spectrometer, and endoproteinase Lys-C peptide mapping was used to obtain sequence data for MBP peptides.

Binding of ^{3}H-DFP and MBP. MS C1 (5 μg) was incubated with increasing amounts of [^{3}H]diisopropyl fluorophosphate (^{3}H-DFP; specific activity 4.06 Ci/mmol; Perkin-Elmer, Woodbridge, Canada) at 37 °C in 10 mM Tris buffer, pH 7.5, for 1 h. Reactions were terminated by the addition of 100 μg of BSA and cold 10% TCA and filtered through glass microfiber filters (GF/B; Whatman, Clifton, NJ), and filters were washed several times with cold 10% TCA. The filters were added to scintillation fluid and counted in a scintillation counter (Beckman LS 6000 IC).

HPLC Analysis of DFP−MS C1. MS C1 (20 μg) and 100 μCi of ^{3}H-DFP were incubated in 10 mM Tris, pH 7.5, at 37 °C for 1 h. The labeled C1 was dialyzed against 500 mL of 100 mM ammonium bicarbonate for 4 h to remove unbound label and lyophilized. The labeled C1 was digested with endoproteinase Lys-C (sequencing grade; Roche, Laval, Canada) (enzyme to substrate ratio, 1:100) in 100 mM ammonium bicarbonate at 37 °C for 18 h. The digest was applied to an LKB C18 HPLC column, and a gradient of solvent A (0.05% trifluoroacetic acid) and solvent B (60% acetonitrile, 40% solvent A) was used. The gradient started at 0% solvent B and increased to 100% solvent B in 50 min and then decreased to 0% solvent B in 10 min. Sixty fractions were collected at 1 min intervals, and the absorbance was monitored at 226 nm. Aliquots of each fraction were counted in a scintillation counter.

Identification of the DFP Binding Site by Mass Spectrometry. MS C1 was modified with 1 mM cold DFP (Calbiochem, San Diego, CA) at 37 °C in 10 mM Tris buffer, pH 7.5, for 1 h. The modified protein was dialyzed against 500 mL of 100 mM ammonium bicarbonate for 4 h and lyophilized. The modified C1 was digested with endoproteinase Lys-C in 100 mM ammonium bicarbonate at 37 °C for 18 h. After the protein was run on HPLC, fractions, corresponding to the two peaks of radioactivity obtained in the HPLC analysis of ^{3}H-DFP-labeled MS C1, were analyzed on a MALDI-QTOF mass spectrometer (Applied Biosystems) to obtain masses of the peptides present. Briefly, the sample to be analyzed was mixed with an equal volume of the matrix (2,5-dihydroxybenzoic acid dissolved in acetonitrile−water, 1:1 v/v). The sample was deposited on the MALDI target, and a 337 nm UV laser was used for ionization of the peptides. For mass measurement, nitrogen was used as the curtain gas. Mass spectrometry was carried out by Dr. Yi-Min She at the Advanced Proteomics Technology Center at the Hospital for Sick Children, Toronto.

RESULTS

MS C1 Undergoes Cleavage and Release of Epitope 84−89 at a Greater Rate than Normal C1. While studying proteolytic cleavage of MBP, we observed that C1 from MS patients (MS C1) was cleaved upon incubation at 37 °C without exogenous enzyme. To compare the rates of cleavage between MS C1 and C1 isolated from normal individuals (N C1), time course experiments were done between 0 and 96 h (Figure 1). N C1 and MS C1 were incubated at 37 °C in buffer (5 mM $CaCl_2$, 50 mM Tris, pH 7.5, 0.02% NaN_3), and at timed intervals, aliquots (2 μg) were removed from the reaction, added to the sample buffer, and frozen. Aliquots

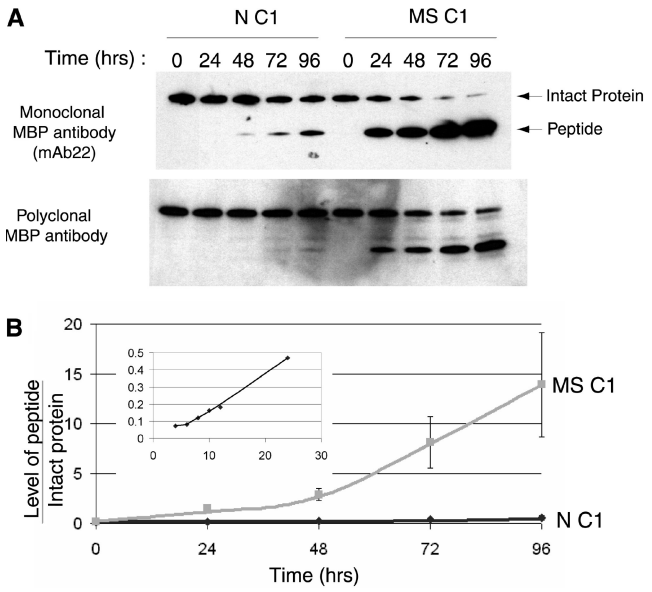

FIGURE 1: MS C1 undergoes cleavage at a faster rate than normal C1. (A) Time course experiments were carried out on N C1 and MS C1 incubated at 37 °C, and Western blots were done. Several cleavage products were observed when a polyclonal MBP antibody was used, but only one major peptide was seen when a monoclonal MBP antibody to epitope 84−89 was used. (B) Western blots were quantitated, and the level of peptide/intact protein was plotted against time. MS C1 showed cleavage and release of epitope 84−89 at a rate faster than that observed for N C1. An earlier time course, run between 0 and 24 h (inset), indicated release of epitope 84−89 at 6 h of incubation. $n = 3$ different MS C1 and N C1 brain samples. Error bars = standard deviation.

were run on 10−20% gradient gels, and Western blots were carried out using both a polyclonal MBP antibody and the monoclonal antibody (mAb22) to the immunodominant region 84−89 of MBP (Figure 1A). While several bands were detected with the polyclonal antibody to MBP, the monoclonal antibody detected the intact protein as well as a peptide released from the intact protein containing the 84−89 epitope.

Both N C1 and MS C1 showed loss of the intact protein and an increase in the peptide containing the 84−89 epitope over the time course examined. MS C1 showed release of this peptide at a much greater rate than N C1. This observation was seen in three different preparations of N C1 and MS C1 obtained from different patients. To determine a more precise time course of the release of epitope 84−89 from MS C1, an earlier time course was run between 0 and 24 h (Figure 1B, inset). Release of epitope 84−89 was first seen at approximately 6 h of incubation at 37 °C and increased steadily up to 24 h. Therefore, upon incubation of N C1 and MS C1 at 37 °C, there is cleavage of the intact protein, with release of a peptide containing the immunodominant epitope of MBP. Since release of this immunodominant epitope would have significance in MS, we further characterized this cleavage.

Cleavage of MS C1 Results from an Intrinsic Property of MBP. To address the issue of contamination of the C1 sample, several experiments were performed. MS C1 was first analyzed by running the protein on a reversed-phase HPLC column (Figure 2A). The HPLC profile showed a single peak indicating a pure sample. The HPLC-purified sample was then tested for cleavage at 37 °C. HPLC-purified MS C1 was cleaved at a similar time course as seen in Figure 1 (data not shown). MS C1 was also analyzed for purity by mass spectrometry (Figure 2B). A single peak at 18499.773 amu represented the intact protein with an acetylated N-terminus which has a predicted mass of 18501.8 amu. Several species at higher masses were also detected. On the basis of the masses observed, these represented adducts of MBP with the matrix material used in the procedure. The matrix, gentisic acid (2,5-dihydroxybenzoic acid), is known to bind basic proteins, such as MBP (Dr. Y. She, personal communication).

In another experiment, 200 μg of MS C1 was incubated at 37 °C for 96 h, and the reaction mixture was fractionated on an HPLC column to separate the peptides from the remaining intact protein (Figure 3A). The HPLC profile showed six main peaks at fractions 1, 3, 6, 8, 14, and 15. On the basis of the known HPLC profile of intact MS C1 (run previously), fraction 15 contained the remaining intact protein from the digest. This fraction was recovered, lyophilized, and reincubated at 37 °C for an additional 96 h. A time course was run, and a Western blot was done with mAb22 (Figure 3B). At time 0, the MS C1 preparation contained some of the peptide 84−89 that likely eluted with the intact fraction from the HPLC column. Upon further incubation, MS C1 continued to undergo cleavage through another 96 h of incubation. Therefore, cleavage of MS C1 seemed to result from an intrinsic activity of the protein, and we therefore believe it to be an autocatalytic mechanism. While we cannot demonstrate definitively the absence of a contaminating protein in the MBP preparation, we believe this is not a factor as the MBP was originally purified by acid extraction followed by ion-exchange chromatography and dialysis.

Characteristics of the Reaction. (a) Effect of Protease Inhibitors. To determine the effect of a range of protease inhibitors on the autocatalytic cleavage of MS C1, the protein was incubated with several protease inhibitors for 24 h (Figure 4A). Concentrations of protease inhibitors were chosen on the basis of the known typical working concentration of each, PMSF (0.1−1 mM), leupeptin (10−100 μM), pepstatin A (1−5 μM), and EDTA (1−10 mM) (Sigma-Aldrich, Technical Bulletin, www.sigmaaldrich.com). The removal of calcium from the buffer, EGTA, and β-mercaptoethanol did not inhibit cleavage. Treatment with leupeptin, which inhibits serine and cysteine proteases, showed reduced cleavage. Pepstatin A, which has activity on aspartic residues, also showed reduced cleavage. However, the amount used for the experiment far exceeded the standard working concentration for this inhibitor, which may explain the reduced activity. The only agents that completely inhibited cleavage were PMSF and the protease inhibitor cocktail (Roche) that contained inhibitors for serine and cysteine proteases. These results suggested that the serine residue(s) was (were) involved in the mechanism of C1 autocatalytic cleavage.

To explore inhibition by PMSF further, MS C1 cleavage was examined with a concentration gradient of the inhibitor (Figure 4B). PMSF showed a concentration-dependent inhibition of the cleavage.

(b) pH Dependence. To further characterize the nature of the autocatalytic cleavage of MS C1, digestions were carried out under conditions of varying pH (Figure 5A). MS C1 incubated for 72 h in buffers of pH varying from 2.0 and

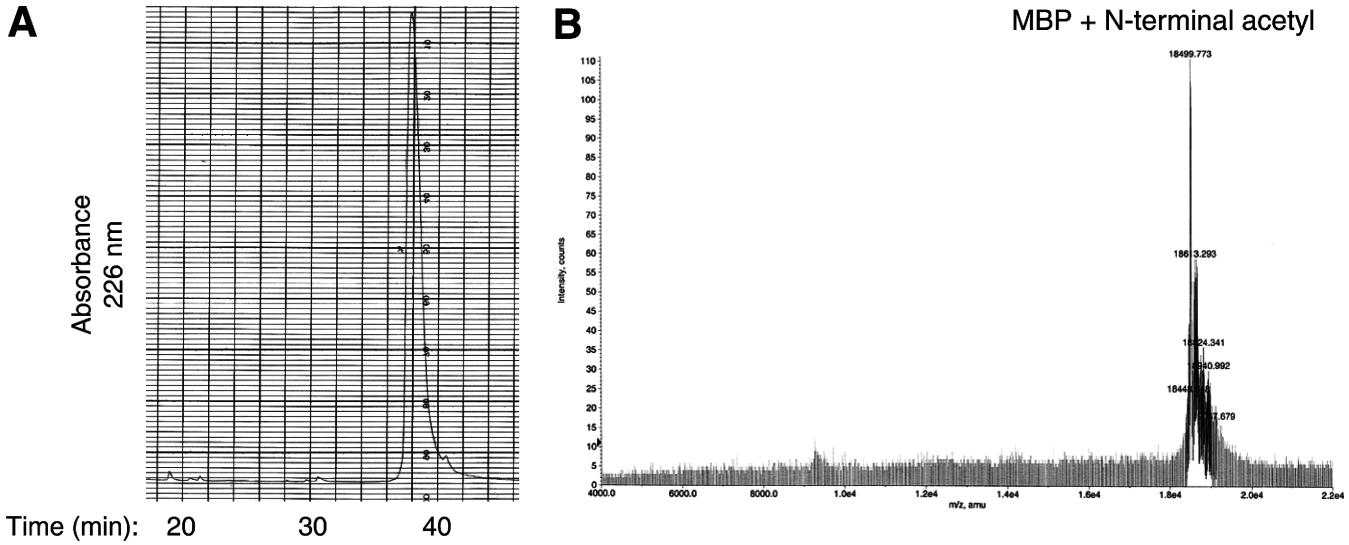

FIGURE 2: Analysis of MS C1 by HPLC and mass spectrometry. MS C1 was run through a C18 reversed-phase HPLC column to monitor for the presence of impurities. MS C1 was also analyzed by mass spectrometry. Both analyses showed a single peak, indicating a pure sample.

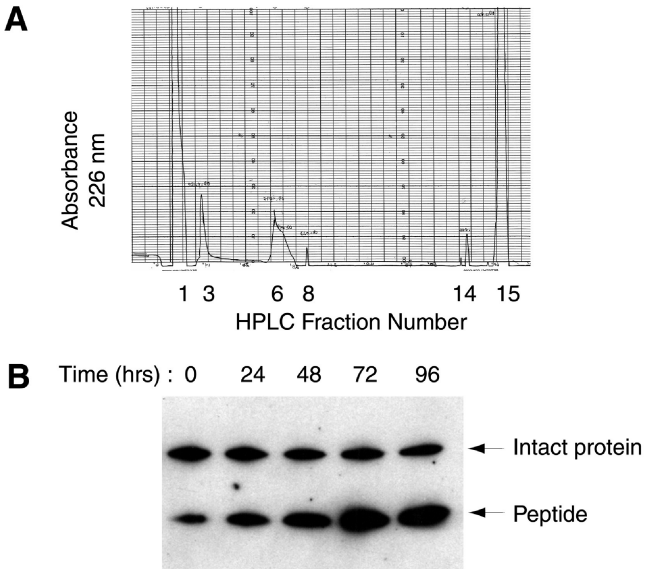

FIGURE 3: Reincubation of HPLC-purified MS C1. (A) MS C1 (200 μg) was incubated for 96 h and run through an HPLC column to separate the intact protein from the peptides released. (B) The fraction containing the intact protein was reincubated for an additional 96 h, and aliquots were run on a gel which was probed with mAb22.

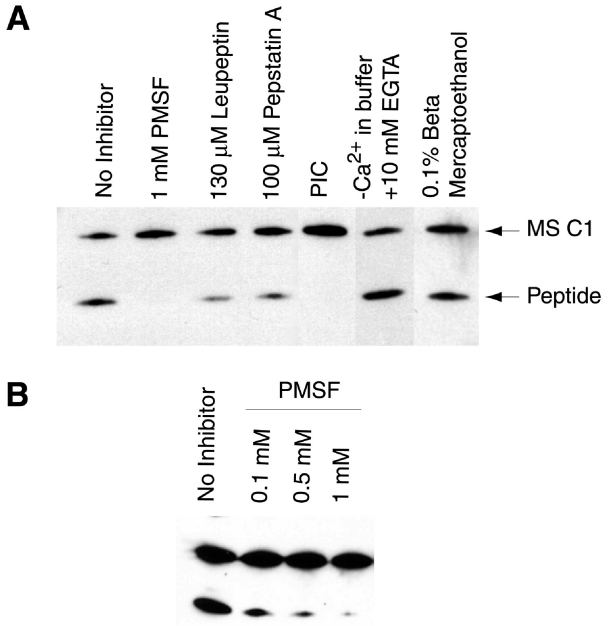

FIGURE 4: Effect of protease inhibitors on cleavage of MS C1. (A) MS C1 was incubated at 37 °C for 24 h under seven different reaction conditions. Lanes (from left): 1, no inhibitor; 2, 1 mM PMSF; 3, 130 μM leupeptin; 4, 100 μM pepstatin A; 5, PIC (protease inhibitor cocktail) for serine and cysteine proteases (Roche); 6, calcium-free buffer plus 10 mM EGTA; 7, 0.1% β-mercaptoethanol. Only PMSF and the protease inhibitor cocktail inhibited the cleavage of MS C1 at 37 °C. (B) MS C1 was incubated at 37 °C for 24 h with different concentrations of PMSF. Inhibition by PMSF was concentration dependent.

9.0 showed release of epitope 84−89 under all conditions tested (Figure 5A,B). Very little release of peptide occurred up to pH 6.0 but became rapid thereafter. For subsequent experiments, we chose to carry out reactions at pH 7.5 since this most closely resembles physiological pH and cleavage was near maximal at this pH.

(c) Temperature Dependence. The temperature dependence of the reaction is shown in Figure 5C. At 4 and 24 °C, no peptide was released after 24 h of incubation. The amount of epitope 84−89 released was greatest at 37 °C, while at 47 and 60 °C, only a small amount of peptide was released. To examine the effect of denaturation on C1 cleavage, MS C1 was boiled for 30 min prior to incubation at 37 °C for 48 h (Figure 5C, last lane). No cleavage was observed, and no peptide was released. Therefore, when MS C1 is denatured by heat, it loses its ability to self-cleave.

(d) Effect of Lipid. To examine if MS C1 would undergo cleavage in the presence of lipid, the protein was incubated at 37 °C with lipid (1:1 mixture of phosphatidyl choline−phosphatidylserine) at a lipid:protein ratio of 2.5:1 to approximate the lipid to protein ratio of myelin (Figure 5D). The presence of lipid seemed to slow the cleavage of MS C1. Higher molecular weight aggregates of MBP could be seen on the gel, likely resulting from binding of MBP to the lipid. Therefore, although MBP was seen to bind to the lipid, the cleavage still occurred, implying that cleavage sites were not masked by lipid.

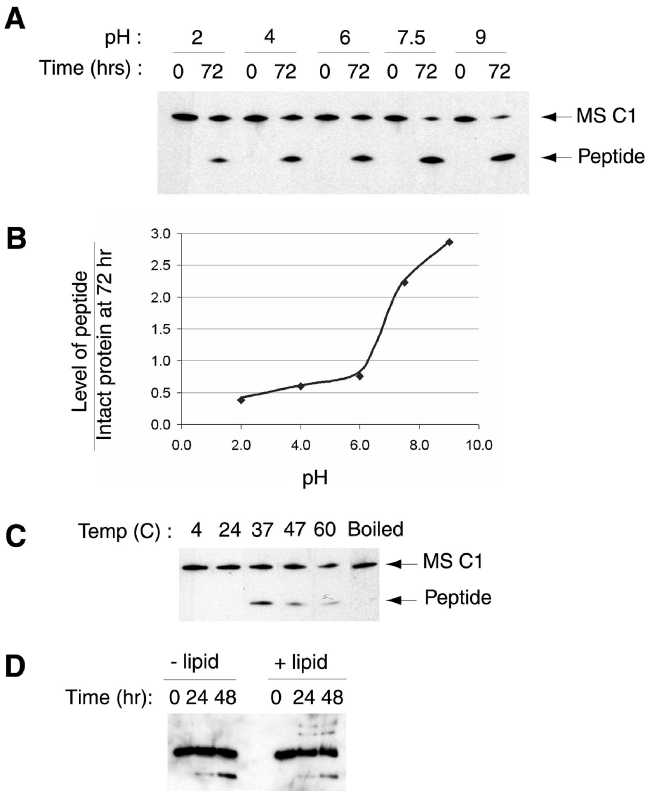

FIGURE 5: Effect of pH, temperature, boiling, and lipids on the autocatalytic cleavage of MS C1. (A) MS C1 was incubated for 72 h in buffers of pH 2.0−9.0. Western blots were done using mAb22 to detect epitope 84−89. (B) Blots were quantitated, and the level of peptide/intact protein was plotted against pH. (C) MS C1 was incubated for 24 h in buffer at pH 7.5 at various temperatures. Boiling MS C1, prior to incubation at 37 °C for 48 h, abolished the autocatalytic cleavage. (D) MS C1 was incubated at 37 °C in the absence and presence of lipid (1:1 mixture of phosphatidylcholine−phosphatidylserine). Aliquots were removed at timed intervals and run on gels which were probed for MBP 84−89 with mAb22.

Autocatalytic Cleavage of Recombinant Mouse MBP. To determine if MBP cleavage was restricted to the human protein, two recombinant murine 18.5 kDa MBP proteins were examined under the same reaction conditions (Figure 6). A recombinant mouse protein representing the least modified charge isomer C1 (termed quasi-C1 or qC1) was used (*13*), as well as a mutant form in which six positively charged residues (five Arg, one Lys) were replaced by Gln. This protein was designed to mimic the less cationic human C8 and is termed qC8 (*14*). Incubation of both proteins at 37 °C resulted in cleavage with release of epitope 84−89 of similar size as previously observed for MS C1 isolated from human white matter. Interestingly, the two recombinant proteins showed different rates of cleavage, with qC8 showing faster cleavage than qC1 (Figure 6B). Therefore, cleavage of MBP is not restricted to the human protein and is not dependent on posttranslational modifications. However, since the two proteins differed in net charge, conformation is likely an important determinant of cleavage rate.

Identification of Peptides Released by Autocleavage. Although cleavage of MS C1 produces one band that reacts with mAb22, use of a polyclonal antibody to MBP showed the presence of several cleavage products (Figure 1A). To better characterize the products of MS C1 autocleavage, mass spectrometry was used to determine individual peptide masses and sequences. MS C1 (60 μg) was digested for 96 h, run on a gel, and stained with Coomassie Blue (Figure 7). Seven bands were identified. A Western blot for epitope 84−89 is shown alongside the Coomassie-stained gel and shows a single band that corresponded to band 4 in the Coomassie Blue-stained gel. Each band in the stained gel was excised from the gel, and the peptides eluted according to Cohen and Chait (*12*). Band 3 was a doublet that ran very close together and could not be separated. Bands 3−7 were analyzed by mass spectrometry as these were most abundant.

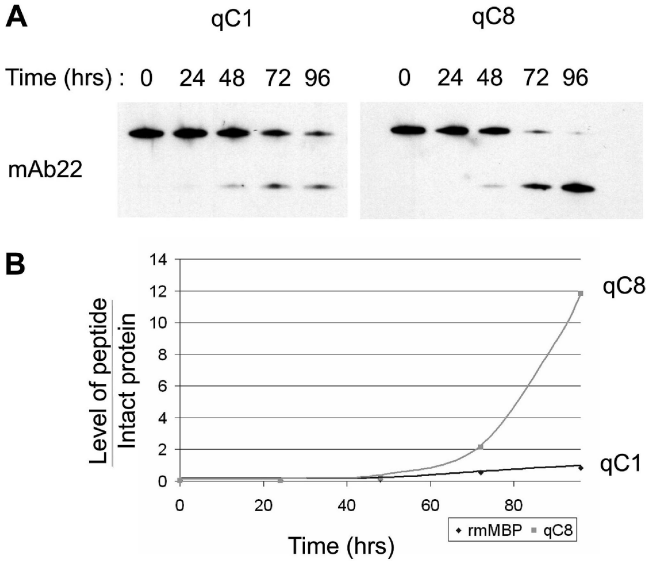

FIGURE 6: Incubation of recombinant MBP proteins. (A) Recombinant mouse MBP proteins were incubated at 37 °C from 0 to 96 h, and aliquots from the incubation were run on gels which were probed with mAb22 to MBP 84−89. A normal recombinant mouse protein (qC1) was used, as well as one containing amino acid substitutions that removed six positive residues (qC8). (B) The level of peptide relative to the level of intact protein is shown for qC1 and qC8.

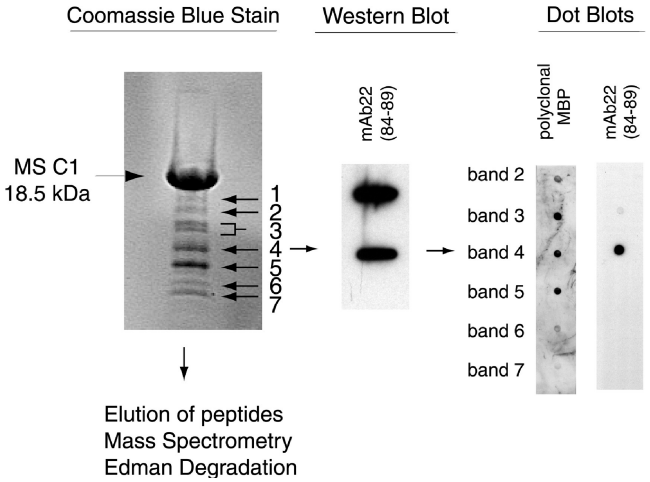

FIGURE 7: Analysis of MS C1 cleavage products. MS C1 was digested for 96 h, run on a gel, and stained with Coomassie Blue and showed seven peptide bands. Bands were eluted from the gel using the method of Cohen and Chait (*12*). Antibody analysis was done on cleavage products extracted and applied onto nitrocellulose membrane using a dot-blot apparatus. A polyclonal antibody to MBP detected all bands extracted. mAb22 only detected band 4, indicating the presence of epitope 84−89.

Table 1: Mass Spectrometric Analysis of MS C1 Autocleavage[a]

band no. extracted from gel	mass (amu) measured	mass (amu) predicted	peptide	confirmed by endo Lys-C peptide mapping	confirmed by N-terminal sequencing
3	12588.392	12589.25	1−114	√	
	11501.77	115000.89	65−170	√	
4	10388.2	10388.67	75−170	√	√
	10011.901	10012.111	1−90	√	
	9864.603	9864.93	1−89	√	
5	8522.396	8523.62	91−170 + methyl		
	8507.208	8509.62	91−170	√	√
	8131.645	8132.05	1−74	√	
6	7020.445	7020.83	1−64		
7	5932.991	5933.61	115−170	√	

[a] The digest, resulting from MS C1 autocleavage, was run on a gel, and the gel was stained (Figure 7). Bands resulting from MS C1 autocleavage were extracted from the gel and analyzed by mass spectrometry. The masses obtained were used to identify MBP peptides.

The masses and sequences obtained are shown in Table 1. Several peptides were identified. Because MBP contains various posttranslational modifications, peptides with various combinations of modifications were found; however, only the major peptides were examined. Methylated and dimethylated species were observed for peptides 1−114, 75−170, and 91−170, since MBP is known to be methylated at Arg107 (*15*). The majority of sequences identified on the basis of mass were confirmed by endoproteinase Lys-C peptide mapping (Table 1). Band 4 contained two peptides with sequences 1−89/90 and 75−170. Amino-terminal sequencing of band 4 by the Edman method gave an N-terminal sequence of KHGRTQ as the only sequence. This corresponds to residues 75−80 and identifies peptide 75−170. Peptide 1−89/90 was not seen by N-terminal sequencing; however, the N-terminus of MBP is known to be acetylated, and the blocked N-terminus is likely responsible for the lack of sequence. Band 5 was also sequenced by the Edman method and gave the sequence KNIVTP as the N-terminus corresponding to residues 91−96. When bands 2 through 7, eluted from the gel, were applied to a dot-blot apparatus (Figure 7), only band 4 reacted with antibody to the immunodominant epitope 84−89.

Modifying MS C1 with DFP Inhibits Autocleavage. Diisopropyl fluorophosphate (DFP) is an organic compound that binds covalently to active site serine residues and has been used extensively in studies of serine proteases (*16*, *17*). Since our experiments showed inhibition of MS C1 autocleavage with serine protease inhibitors (Figure 4), this suggested that a serine residue was involved in the autocatalytic mechanism. We therefore used DFP to modify MS C1 and identify the active serine involved.

To examine whether DFP would bind to MS C1, it was incubated with increasing amounts of ^{3}H-DFP at 37 °C for 1 h (Figure 8A). MS C1 was labeled with ^{3}H-DFP, and maximum labeling was achieved when 6−12 nmol of ^{3}H-DFP was used.

To test whether DFP would inhibit autocleavage of C1, MS C1 was labeled with an excess of cold DFP and dialyzed to remove unbound label. The DFP-modified C1 was incubated at 37 °C from 0 to 96 h. At timed intervals, 2 μg

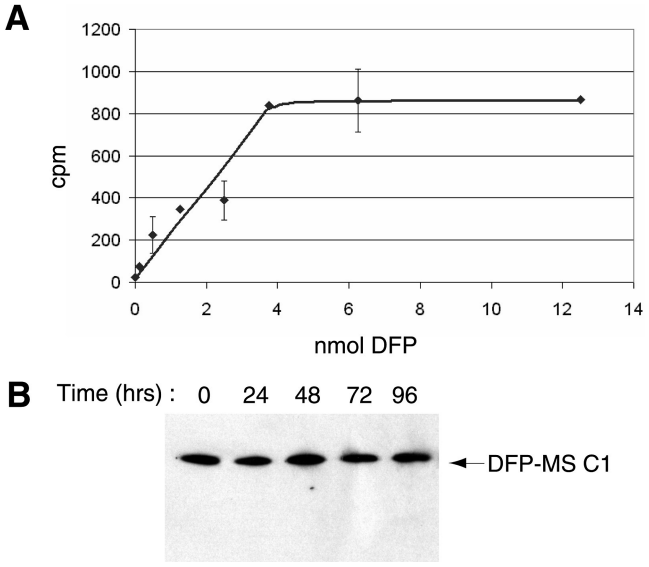

FIGURE 8: DFP binds to MS C1 and inhibits MS C1 autocleavage. (A) MS C1 was incubated with increasing amounts of ^{3}H-DFP. The protein was precipitated and applied to glass fiber filters which were washed and counted in a scinitillation counter. ^{3}H-DFP was able to bind to MS C1, and 6−12 nmol of DFP was required to achieve maximum binding. The standard deviation is shown from three independent experiments. (B) DFP-labeled MS C1 was incubated at 37 °C to test for autocleavage. A Western blot was done on aliquots from the reaction using mAb22. DFP labeling inhibited MS C1 autodigestion, implicating a serine residue as a critical residue in the reaction.

aliquots were removed, added to the sample buffer, and frozen on dry ice. Aliquots were run on 10−20% gradient gels, and a Western blot was done with mAb22 (Figure 8B). DFP inhibited the cleavage of MS C1 previously observed, indicating that the modified serine of MS C1 was necessary for the autocatalytic reaction.

Identification of Active Serine. To identify the site(s) that were modified, MS C1 was labeled with ^{3}H-DFP and dialyzed to remove unbound label. The labeled protein was digested with endoproteinase Lys-C (sequencing grade; Roche), and the resulting peptides were separated on an HPLC column. Sixty fractions were collected, and an aliquot of each fraction was counted in a scintillation counter (Figure 9A). A major peak of radioactivity occurred in fraction 30, while a minor peak occurred at fraction 16. Because we could not find any mass spectrometry service that would analyze a radiolabeled sample, the experiment was repeated with cold DFP to identify the endo Lys-C peptide that was labeled.

Figure 9B shows the HPLC profile of the endo Lys-C digest of DFP−MS C1. Since fractions 15−17 and 29−31 gave the highest counts when ^{3}H-DFP was used, these fractions were analyzed further by mass spectrometry. The masses of the peptides in each fraction were obtained (data not shown) and compared to masses predicted for endo Lys-C digestion of the unlabeled protein (Protein Prospector/MS-Digest search program at http://prospector.ucsf.edu). The presence of posttranslational modifications was also taken into consideration using the known mass shifts each modification would add to the peptide. For example, phosphorylation would add 80 amu.

Fractions 15−17 contained species of very small mass (600−800 amu). The radioactive peak observed in these fractions may represent breakdown products of ^{3}H-DFP as

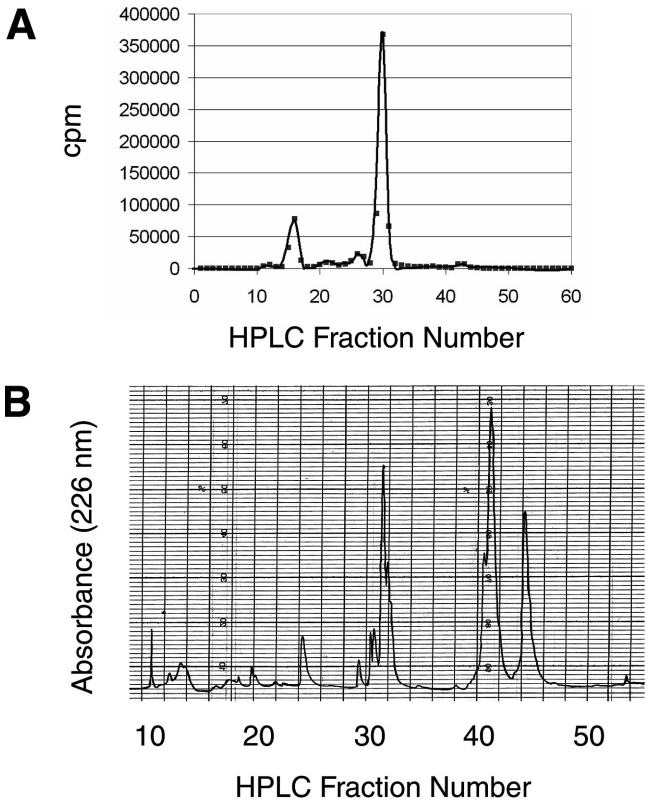

FIGURE 9: HPLC analysis of DFP−MS C1. (A) To identify the labeled site, ^{3}H-DFP-labeled MS C1 was digested with endoproteinase Lys-C, and cleavage products were run on an HPLC column. An aliquot of each fraction was counted in a liquid scintillation counter. Fraction 30 gave the highest counts per minute (cpm). (B) The experiment was repeated with nonradioactive DFP, and the HPLC profile for the endo Lys-C digest is shown.

has been suggested by other researchers (*18*). Fractions 29 and 31 contained MBP peptides whose masses indicated that they were not labeled with DFP (data not shown).

Fraction 30 contained three major species when analyzed by mass spectrometry (Figure 10). The major peptide had a mass of 1052.572 which corresponded to peptide ^{5}RPSQRHGSK^{13} which has a predicted mass of 1052.5713 (Table 2). A peptide with a mass of 1551.815 was also observed in fraction 30. The predicted mass of the peptide ^{140}GFKGVDAQGTLSK^{152}, with one phosphorylation, was 1387.6623. A peptide with a mass of 1387.719 amu was observed in fraction 30 at low intensity (Table 2). The addition of DFP to a serine residue in this peptide would add 164.2 amu after loss of the serine hydrogen (1 amu). Therefore, the mass of this peptide, labeled with DFP, would be 1551.8623. Therefore, peptide 140−152 was likely the labeled peptide. Because this peptide had only one serine residue, Ser151 must be the labeled serine.

DISCUSSION

MS has traditionally been regarded as an autoimmune disease in which autoreactive T-cells attack myelin components and initiate demyelination. Molecular mimicry is a popular mechanism by which viruses are believed to activate the autoimmune response (*3*, *19*). This mechanism has gained support from studies in which activation of autoreactive T-cells follow immunization of animals with viral sequences sharing sequence homology to self-peptides (*3*, *20*). However, it is known that linear sequence homology is not critical in T-cell recognition, as structural homology is also important (*6*, *21*). In addition to molecular mimicry, there are other mechanisms by which viruses may contribute to autoimmune disease, for example, by induction of an inflammatory response or by tissue damage of the infected organ. At present, the role of viruses in MS pathology remains unclear.

Although we do not claim that molecular mimicry is not a factor in MS, our results show an alternative mechanism for the generation of the immunodominant epitope of MBP. In the present study, we have observed an important difference in the behavior of MBP isolated from normal individuals and patients with MS. Incubation of C1 from MS patients (MS C1) at 37 °C resulted in a characteristic and reproducible cleavage of the protein into several distinct bands, including the immunodominant epitope 84−89, which was stable up to 96 h of incubation. This peptide may undergo further processing and be carried out into the periphery where interaction with cells of the immune system may subsequently prompt an autoimmune attack. Autocleav-

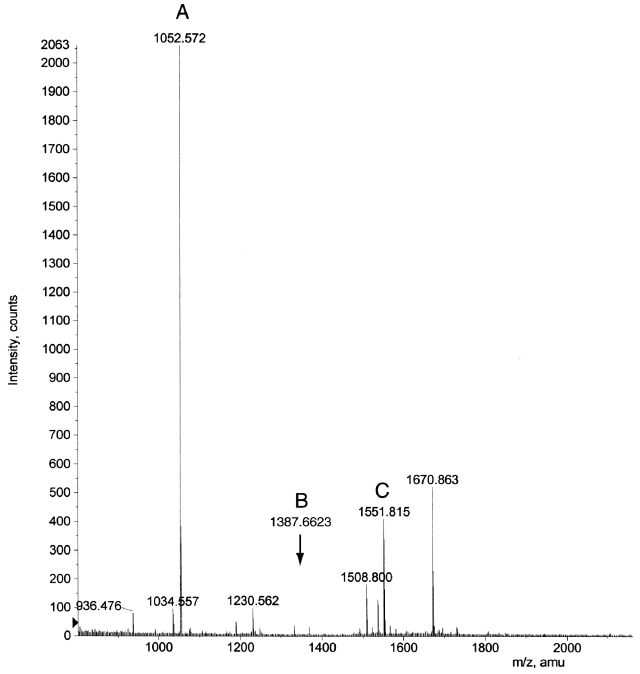

FIGURE 10: Identification of the labeled serine by mass spectrometry. Fraction 30, obtained from the HPLC analysis of DFP−MS C1 (Figure 9), was analyzed by mass spectrometry. Peak C was found to be the adduct with DFP.

Table 2: Identification of DFP-Modified MBP Peptide by Mass Spectrometry[a]

	peptide	mass (amu) predicted	mass (amu) measured
A	^{5}RPSQRHGSK^{13}	1052.5713	1052.572
B	^{140}GFKGVDAQGTLSK^{152} + PO_4	1387.6623	1387.719 (low intensity)
C	^{140}GFKGVDAQGTLSK^{152} + PO_4 + DFP	1551.8623	1551.815

[a] DFP-labeled MS C1 was digested with endoproteinase Lys-C, and cleavage products were run on an HPLC column (Figure 9B). Fraction 30 was analyzed by mass spectrometry. By comparing the masses of the products obtained in fraction 30 with the predicted masses of endoproteinase Lys-C peptides, a modified peptide was identified consisting of residues 140−152.

age of MBP and release of the immunodominant epitope provide a mechanism for activation of the immune response in MS.

While we cannot demonstrate definitively the absence of a contaminating protein in the MBP preparation, we believe this is not a factor in the cleavage reaction. Cleavage occurred with HPLC-purified MBP, and no contamination of the sample was identified by mass spectrometry. In addition, each charge isomer of MBP (C1, C2, C3, C4, C5, and C8) isolated with cation-exchange chromatography demonstrated cleavage upon incubation at 37 °C (data not shown).

In the initial isolation of MBP, the tissue was extracted with chloroform−methanol and acid and fractionated by ion-exchange chromatography in urea. These treatments would dissociate any tightly bound proteins. While MBP is denatured in the process, it likely refolds upon removal of denaturants and salt by dialysis. Structural analysis of bovine MBP, isolated using the same protocol used in this study, showed that its conformation did not significantly differ from a recombinant mouse MBP (qC1) purified without use of organic solvents or acid (*13*). Circular dichroic (CD) spectroscopy showed that both proteins had a mostly random coil conformation in aqueous solution and acquired an increase in secondary structure (α-helix and β-sheet) in the presence of lipid. In the present study, this recombinant protein (qC1) also displayed autocleavage, suggesting that both MS C1, purified under harsher conditions, and the recombinant mouse protein possessed the appropriate conformation for cleavage.

The autocleavage of qC1 and qC8 in this study occurred at different rates (Figure 6B), with qC8 showing faster cleavage. Structural analysis of the two proteins by CD spectroscopy showed that qC8 was in a more open conformation than qC1 in aqueous solution (*14*). Deimination has been shown to increase the proportion of random coil structure in MBP (*22*). Since the mutant residues in qC8 were chosen to mimic the effects of deimination, its increased rate of autocleavage is likely due to an altered, more open conformation. Because cleavage also occurred in recombinant MBP proteins, this suggests that posttranslational modifications are not a requirement for cleavage to occur. However, qC1 and qC8 differ in net charge and have different conformations (*14*). Therefore, the conformation of MBP likely affects the rate at which cleavage of MBP occurs.

Although we do not have an explanation for the different cleavage rates of N C1 and MS C1, the difference likely reflects differences in local secondary structure between the two proteins. Sequence analysis by mass spectrometry (data not shown) revealed no difference in primary structure between N C1 and MS C1. Therefore, the different rates of cleavage may result from different posttranslational modifications and conformations. Posttranslational modifications have been shown to alter the conformation of MBP (*22*−*24*). For example, phosphorylation has been reported to increase and stabilize the β-structure in MBP through interaction of the phosphate group with nearby basic residues (*24*). In a recent paper by Kim et al. (*15*), MS C1 was shown to be less phosphorylated than C1 isolated from normal individuals. Therefore, the increased secondary structure associated with phosphorylation in the normal protein may diminish its rate of autocleavage.

MBP autocleavage was shown to occur in the presence of lipids. In MS myelin, the presence of increased levels of C8 is thought to destabilize myelin and loosen compaction. Therefore, this instability of MS myelin may enhance the rate of autocleavage in vivo. However, in normal individuals, a stable, compact myelin sheath may prevent autocleavage of MBP from occurring at all.

Conformational differences between proteins with identical primary sequence have been shown to result in functional differences. For example, in transmissible spongiform encephalopathies, disease has been attributed to prions, which are proteinaceous particles with a pathogenic conformation (*25*). The normal version of the prion protein does not cause disease. The two proteins differ in their conformation, with the pathogenic protein having high β-sheet content, while the normal protein has a higher α-helical content. Conformation of MS C1 likely plays an important role in its autocatalytic activity.

Our experiments with protease inhibitors suggested that serine residues were important to the mechanism of C1 cleavage. To identify the active serine residue in MBP, MS C1 was labeled with diisopropyl fluorophosphate (DFP). DFP is a protease inhibitor that reacts selectively with serine residues. It does not normally react with the hydroxyl group of serine unless the serine is activated; i.e., its hydroxyl group is hydrogen bonded to a side chain of another residue. In this structure, the serine has a nucleophilic oxygen atom and can "attack" and cleave a peptide bond. We were able to label Ser151 of MS C1 with DFP, indicating an activated serine residue. In addition, labeling of MS C1 with DFP abolished the autocatalytic cleavage previously observed upon incubation at 37 °C (Figure 8B).

A protein that exhibits similar properties to the observations in this study is the LexA repressor protein of *Escherichia coli* (*26*), which is involved in regulating a set of genes involved in the SOS response to DNA damage. LexA undergoes autodigestion at high pH via a mechanism involving a nucleophilic attack by a serine residue, analogous to serine proteases (*27*,*28*). Studies have shown that Ser119 acts as a nucleophile that hydrolyzes the peptide bond, while a deprotonated lysine residue (Lys156) activates the Ser119 hydroxyl group. In addition, Ser119 was the only serine to react with DFP (*18*).

The work presented here supports the notion that myelin breakdown is a primary event in MS pathogenesis. Autocatalysis of MBP allows release of peptides for subsequent activation of T-cells in the periphery. In an extensive review of the literature, Behan et al. (*29*) noted that early researchers reported myelin destruction in MS in the absence of inflammatory cells. In addition, many cases of MS showed minimal inflammation upon histological examination (*30*). Several researchers believed that the myelin sheath was affected first, followed by lymphocyte invasion (*29*).

Thus, we propose that there are inherent abnormalities in myelin from MS patients. We believe that the altered conformation of MBP from MS patients results in autocatalysis. Breakdown of MBP and, consequently, myelin integrity would result in a primary demyelinating event that is followed by an autoimmune attack which further propagates the disease.

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