Rapid Release and Unusual Stability of Immunodominant Peptide 45−89 from Citrullinated Myelin Basic Protein†

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ABSTRACT: Myelin basic protein (MBP) exists in a population of isoforms and isomers. The 18.5 kDa MBP-C1, the main human adult isoform, has 170 residues and is relatively unmodified, whereas the same isoform can be citrullinated on six arginine residues to create the MBP-C8 (MBP Cit_6) isomer. MBP Cit_6 dominates in MS brain, accounting for 45% rather than 25% of the population of MBP isomers. In the fulminant form of MS, known as Marburg's Disease, 18 of the 19 arginines in MBP are citrullinated (MBP Cit_{18}). Citrullination of MBP could lead to instability of myelin or limited remyelination. In this investigation, the susceptibilities to degradation by cathepsin D of MBP Cit_6 and MBP-C1, both from normal and MS brain tissue, and Marburg MBP Cit_{18} were compared. The pattern of digestion was similar, and no differences of corresponding isomers in normal and MS brain were noted. However, normal MBP Cit_6 was degraded 10-fold more rapidly than MBP-C1, and MBP Cit_{18} was degraded even more rapidly. MBP peptide 45−89 was preserved regardless of isomer type or source. Its generation was directly related to the citrulline content of the MBP substrate being 4 times faster in normal MBP Cit_6 and 35 times faster in Marburg MBP Cit_{18} than in normal MBP-C1. Peptide 45−89 from a citrullinated MBP exhibited more deamidation, and, regardless of source, showed an α-helix structure in a lipid mimetic environment. We postulate that the generation of MBP peptides, including those that are dominant and encephalitogenic, is directly related to deimination of arginine to citrulline in MBP.

Although the etiology of MS[1] is unknown, genetic, environmental, and immune processes join in concert to produce a complex mechanism leading to demyelination. A T cell-mediated immune response is believed to result from sensitization of lymphocytes with a myelin antigen which may be a myelin protein or a peptide derived from one of the proteins. The origin of these sensitized T cells in MS is unclear, since myelin breakdown would be a necessary prerequisite for generating encephalitogenic peptides. Although molecular mimicry has been suggested as a possible source of initial encephalitogenic peptides, it is nonspecific since several viral peptides may stimulate T cell clones to the immunodominant myelin basic protein (MBP) peptide 85−99 (*1*). Furthermore, EAE induction of experimental allergic encephalomyelitis (EAE) requires Freund's adjuvant, which may protect the peptide against enzymic degradation. Therefore, the problem of the source and nature of the antigen presented initially to the immune system remains.

The proponents of the immunological mechanisms and molecular mimicry propose that myelin destruction occurs after sensitization of T cells which migrate into the central nervous system (CNS); i.e., perivascular lymphocytic infiltration precedes demyelination (*2*). This view is supported by studies with the animal model, EAE, in which lymphocytic infiltration is a prominent feature (*3*). However, unlike EAE, no direct evidence of migration of lymphocytes in MS was evident in some cases of MS (*3*). An alternate explanation is that the primary event is CNS myelin breakdown in which encephalitogenic peptides are released to activate the immune system. This view arises from our chemical studies of the nature of MBP in post-mortem MS brain tissue in which the citrullinated MBP is prominent.

MBP is a family of isoforms (*4*) and isomers (*5*). It is encoded by a single (*6*) or larger Golli-MBP (*7*) gene on chromosome 18. Alternate splicing of MBP mRNA results in several isoforms, and extensive post-translational modifications lead to the generation of multiple charge isomers. Of the isomers, the citrullinated (*8*) one is the most modified and least cationic. The level of citrullinated isomer, designated as MBP-C8, is increased 2−3-fold in chronic MS (*9*), and the isomer contains six citrulline residues (MBP Cit_6).

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[1] Abbreviations: MBP-C1, component 1, the most cationic and least modified, containing 19 Arg residues; MBP Cit_6, component 8 containing 6 citrulline residues and 13 Arg residues; MBP Cit_{18}, component 8 from Marburg's disease containing 18 citrulline residues and 1 Arg residue; MS, multiple sclerosis.

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It accounted for almost 100% of the MBP in fulminating MS of the Marburg type, and 18 of the 19 arginyl residues of MBP were deiminated to citrulline (*10*) (MBP Cit_{18}). Since the level of citrullinated MBP was not found to be increased in acute or chronic EAE (*11*), or the result of post-mortem degradation (*9*), the increased levels of MBP-C8 detected in MS are unlikely to be an artifact.

Immunoreactive MBP peptides have been detected in cerebrospinal fluid (*12*) and urine (*13*) of patients with MS. The source of this immunoreactive material is unknown, but it has been suggested that digestion of MBP by cathepsin D, a proteinase which cleaves peptides at Phe−Phe linkages (*14*), may be involved. The level of cathepsin D is elevated in MS brain tissue (*15*). Both invading macrophages (*16*) and reactive astrocytes (*17*) have been shown to produce cathepsin D. Therefore, cathepsin D appears to be abundantly available in the chemical pathogenesis of MS.

In this study, we demonstrate that MBP Cit_6, the citrullinated isomer of MBP (*8*), is unusually susceptible to degradation by cathepsin D in vitro. Furthermore, the rapidity of digestion correlates with the amount of citrulline present in the molecule: the greater the amount of citrulline, the more rapid the digestion. MBP peptide 45−89, which contains an immunodominant epitope in MBP, was shown to be more stable than other MBP peptides released by cathepsin D. We suggest that deimination of arginyl residues of MBP enhances its enzymatic degradation which then generates this, and possibly other, immunodominant peptides.

MATERIALS AND METHODS

Preparation of MBP and Cathepsin D. The charge isomers are formed by post-translational modifications. Component 1 (C1), the most unmodified, contains two post-translational modifications: acylation at the N-terminus and methylated arginine at residue 107. Component 2 (C2) contains one less positive charge than C1, the result of a single deamidation; component 3 (C3) contains two less positive charges than C1 from two deamidations or one phosphorylation and so on for other components. The citrullinated component (C8) contains six citrullinyl residues, the result of deimination of six arginyl residues. Since citrulline is neutral, each deimination of an arginyl residue decreases the positive charge by one. Therefore, C8 found in MBP from normal individuals contains six less positive charges than C1. Since the post-translational modifications result in the loss of positive charge, they are called "charge isomers", although they are not isomers in the mass spectrometric sense. Several of these charge isomers can be separated on CM-52 cation exchange columns in urea. The most unmodified C1 is the most positive and is eluted last from the column, preceded by C2, C3, etc.

MBP was isolated from post-mortem human brain tissue obtained from persons who died of non-neurological causes (normal brain) and those who died with MS, including the rapid variant or Marburg's disease (*10*). The C1 and C8 charge isomers were purified by ion exchange chromatography followed by high-performance liquid chromatography (HPLC) (*8*, *14*). Bovine brain cathepsin D used for MBP digestion was prepared as described previously (*18*) or purchased from a commercial source (Sigma, St. Louis, MO). On the basis of the HPLC profile of the generated peptides, there was no difference in the MBP cleavage sites with enzymes obtained from either of these two sources. Peptides submitted for analysis (see below) all came from fragments generated with the commercial proteinase.

Antibodies and Immunoassay. Antibodies that are reactive with different MBP peptides, for both the C1 and C8 isomers, have been prepared previously. These included F23, a monoclonal antibody (mAb) to MBP peptide acetyl 1−9 (*19*), 845D3, an mAb to human MBP peptide 80−89 (*20*), and R157, a rabbit polyclonal antibody to human MBP peptide 90−170 (*21*). These reagents were used in an ELISA (*22*) to detect and identify MBP peptides in fractions obtained from HPLC of cathepsin D digests. ELISA plates were coated with aliquots of each fraction by first diluting the fraction to a projected concentration of 4 μg/mL with 0.01 M phosphate-buffered saline at pH 7.2 (PBS), washing with PBS, and blocking with 100 μL of 1% bovine serum albumin (w/v) for 1 h. The first antibody was added and remained for 16 h at 4 °C. After the mixture had been washed with PBS, the second antibody, alkaline phosphatase-labeled and appropriate for detection of the first antibody, was added and left for 4 h at 25 °C. The enzyme-labeled goat anti-murine Ig and goat anti-rabbit IgG were commercially obtained (Southern Biotechnology, Birmingham, AL). After the mixture had been washed with PBS, the substrate was added and plates were allowed to develop for approximately 25 min prior to reading at 405 nm on a Titertek Multiscan MCC plate reader manufactured by Flow Laboratories (McLean, VA).

Digestion of MBP by Cathepsin D. Digestions were performed at 37 °C in 0.05 M ammonium acetate buffer (pH 3.5). An MBP concentration of 2.18×10^{-5} M, or 400 μg/mL, was used except in experiments in which different substrate concentrations were used. Enzyme:substrate ratios from 1:50 to 1:500 were investigated. Enzyme reactions were terminated by boiling to inactivate the cathepsin D. The samples were then frozen and lyophilized prior to HPLC.

HPLC. HPLC was conducted on a Waters (Milford, MA) HPLC system equipped with two pumps, a WISP 710 autosampler, a model 450 variable-wavelength UV absorbance detector, and a Digital Equipment Corp. (Maynard, MA) model 380 data system with Waters 840 software. Fractions were collected with an LKB (Bromma, Sweden) 2211 "Superrac" fraction collector. Measurements of pH were taken with a Metrohm/Brinkman (Westbury, NY) pH-104 meter. All HPLC separations were carried out using Vydac (Hesperia, CA) C-18 reverse phase columns and 0.1% trifluoroacetic acid as solvent A and 90% acetonitrile in solvent A as the organic modifier.

HPLC, monitored by absorbance at 210 nM, was developed with a complex gradient method for the analytical scale (240 mm × 4.6 mm) column that was used. The stepwise gradient elution (flow rate of 1 mL/min) was as follows:

time (min)	% A	% B
0	100	0
10	100	0
15	81	19
50	64	36
55	25	75

Electrospray Mass Spectrometry (ESMS). ESMS was performed on a SCIEX instrument API-III triple-quadruple

mass spectrometer (Thornhill, ON). The MBP fragments from cathepsin D digestion were isolated by HPLC and subjected to ESMS for measurement of the molecular mass. The samples were dissolved in water and introduced into the mass spectrometer by flow injection with acetonitrile in water (1:1, v/v) containing 1% acetic acid and 1 mM ammonium acetate at a flow rate of 20 μL/min.

Amino Acid Analysis. The HPLC-purified MBP fragments were subjected to hydrolysis under vacuum in 5.7 N HCl at 110 °C overnight in the gas phase. Amino acid analyses were then carried out using the Waters PicoTag system.

Circular Dichroism. Circular dichroism (CD) spectra were measured on a JASCO-720 spectropolarimeter over the range of 250−190 nm. Spectra were accumulated over 2500 data points using the following conditions: a sensitivity of 20 mdeg, a response of 1 s, a scanning speed of 50 nm/min, and a path length of 0.1 cm at 25 °C. Mean residue ellipticities (θ) (in deg cm^2 dmol^{-1}) were calculated using the equation $\theta = \theta_{obs}(MRW)d^{-1}c^{-1} \times 10^{-1}$, where θ_{obs} is the ellipticity measured in degrees, MRW is the mean residue mass, taken as 108.9 Da, c^{-1} is the concentration of peptide, and d is the optical path length (0.1 cm). Calibration was conducted in 0.06% ammonium-d_{10} camphorsulfonate at 295 nm. Peptide concentrations were determined by amino acid analyses.

RESULTS

Time Course of Digestion of MBP-C1 and MBP Cit$_6$ from Normal Brain and MBP Cit$_{18}$ from Marburg's Disease Brain. The time course of digestion of MBP-C1 with cathepsin D is shown in Figure 1A. Similar profiles were obtained for normal MBP Cit$_6$ and Marburg MBP Cit$_{18}$ (Figure 1B). Cathepsin D digests proteins at Phe−Phe linkages. Since MBP has two such linkages between residues 44 and 45, and 89 and 90, three peptides will be generated, i.e., 1−44, 45−89, and 90−170. Additional peptides such as 45−170 may also be found especially at early times, the result of sequential, partial cleavages (*14*). The digestion of normal MBP Cit$_6$ generated an HPLC profile similar to that of MBP-C1, except that the rates of digestion were faster (data not shown). With MBP Cit$_{18}$, peptide 45−89 was found within 30 min of digestion (Figure 1B), but an equivalent degree of degradation was not present by 60 min for MBP-C1 (Figure 1A).

The MBP-C1 peptides were also identified with specific antibodies (data not shown). The N-terminal peptide was detected with mAb F23C6 specific for acetyl 1−9; MBP peptide 45−89 was detected with mAb 845D3 and MBP peptide 90−170 with a polyclonal anti-MBP antibody. The identity of the peptides was confirmed by amino acid analyses (data not shown).

There are several notable features. (1) Complete digestion of MBP-C1 requires about 360 min, at which time a very small amount of intact MBP is still present (peak marked 1−170). (2) Peptide 45−89 is the last of the peptides to appear, significant amounts of which are found at 60 min. (3) The major peptides, 1−44, 45−89, 90−170, and 45−170, are not degraded further even after 360 min. Since 45−170 was present after digestion for 360 min, the Phe−Phe linkage between residues 89 and 90 in MBP-C1 is more refractory with respect to digestion. Similar observations have been made previously (*14*).

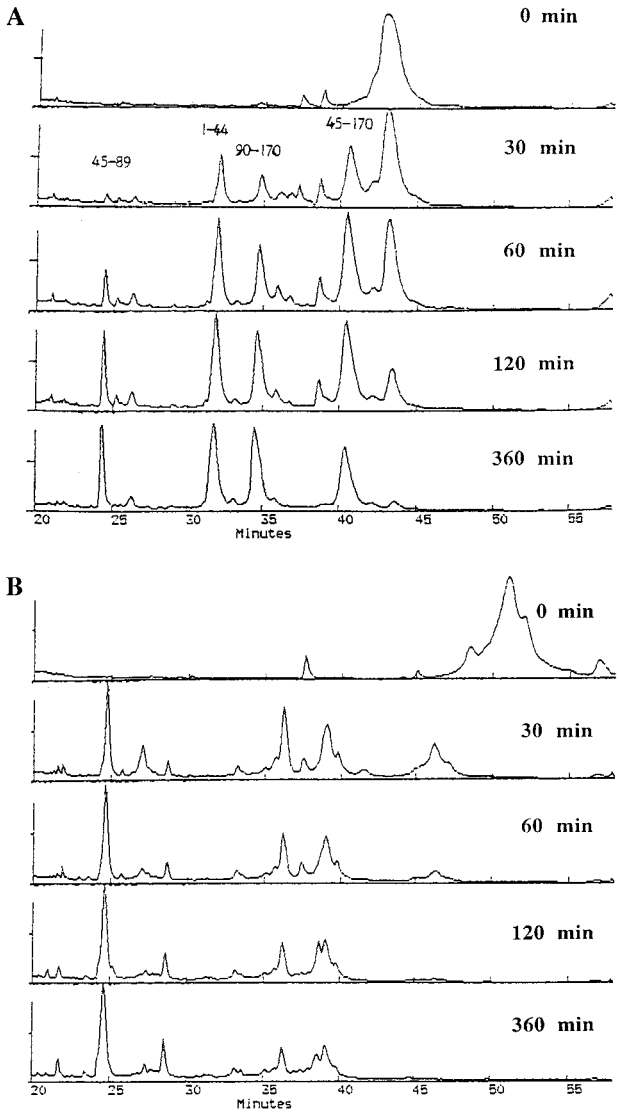

FIGURE 1: Cathepsin D digests of normal MBP-C1 and Marburg MBP Cit$_{18}$. Each was digested with cathepsin D for periods of up to 360 min as described in Materials and Methods. At the end of the digestion times, the sample was lyophilized to remove ammonium acetate, dissolved in solvent (method I), and applied to a C18 HPLC column (4.6 mm × 240 mm). Retention times of known MBP peptides are indicated, from left to right, as 45−89, 1−44, 90−170, and 45−170. The undigested MBP is designated as 1−170: (A) digestion of MBP-C1 and (B) digestion of Marburg MBP Cit$_{18}$.

The digestion of Marburg MBP Cit$_{18}$ is shown in Figure 1B. From an examination of these chromatograms, it is clear that the digestion was much more rapid than with MBP-C1 with none of the MBP Cit$_{18}$ remaining after 30 min. MBP peptides 45−89, 90−170, and 1−44 accounted for most of the chromatogram except for a small amount of MBP peptide 45−170.

The MBP digestion is graphically represented in Figure 2 by plotting the concentration of intact MBP (the area under the HPLC peak) against the time of digestion. Approximate rates of digestion were calculated and are listed in Table 1. Marburg MBP Cit$_{18}$ was digested about 10 times more rapidly than normal MBP-C1. We have also calculated the rate of appearance for MBP peptide 45−89 for normal MBP-C1, normal MBP Cit$_6$, and Marburg MBP Cit$_{18}$. MBP peptide 45−89 from normal MBP Cit$_6$ appeared 4 times more

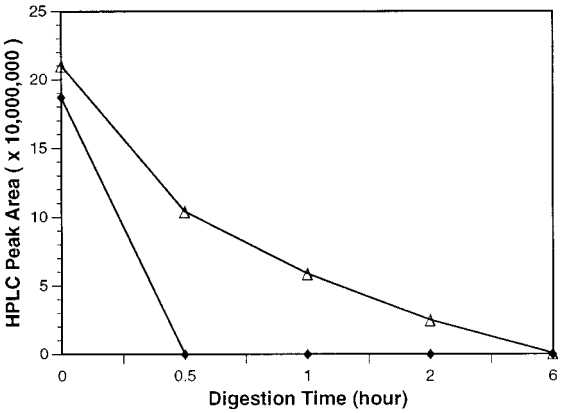

FIGURE 2: Digestion of the normal MBP-C1 and Marburg MBP Cit_{18}. The areas under the peak corresponding to the intact MBP (1−170) from panels A and B of Figure 1 were summed and plotted against the time of digestion for MBP-C1 (△) and MBP Cit_{18} (♦). The Marburg sample was completely digested within 30 min.

Table 1: Digestion of MBP-C1 and MBP-C8 from Normal MBP (N) and MBP-C8 from Marburg's Disease (M)

component	rate of protein digestion	rate of appearance of peptide 45−89[a]	no. of citrullines per mole
C1 (N)	3.5	0.02	0
C8 (N)		0.08	6
C8 (M)	37.0	0.70	18

[a] Rates were computed from the area under the chromatographic peaks divided by the time.

quickly than that from normal MBP-C1, whereas Marburg MBP Cit_{18} peptide 45−89 appeared 35 times more rapidly than the same peptide from MBP-C1. These approximate rates are correlated with the amount of citrulline in the MBP isomer. The increased rate of disappearance of the intact MBP and the rate of appearance of MBP peptide 45−89 were observed with increased amounts of citrulline in the protein.

Digestion of the MBP Cit_{18} from Marburg's Disease Brain. Because the digestion of Marburg MBP Cit_{18} was rapid (Figure 1B), this digestion was examined in more detail at earlier time points of 0−30 min (data not shown). It can be seen that all of the Marburg MBP Cit_{18} was digested by 5 min, at which time peptides 1−44 and 45−170 represent the dominant species. The initial cleavage site is the same as that for the bovine MBP-C1 for cathepsin D (*14*). A trace amount of Marburg MBP Cit_{18} peptide 45−89 is present at 5 min, but the amount increases rapidly after longer periods of digestion. Only a trace of peptide 45−170 remained after 30 min.

When the appearance of Marburg MBP Cit_{18} peptide 45−89 was plotted as a function of time, a linear curve was obtained between 10 and 30 min (Figure 3A), from which the slope gave the rate shown in Table 1. It was clear from these data that the deimination of 18 of 19 arginyl residues to citrullinyl residues enhanced the ability of this MBP isomer to be digested by cathepsin D and that Marburg MBP Cit_{18} peptide 45−89 was not further degraded whereas peptide 45−170 was extensively degraded.

Since MBP peptide 45−89 contains immunodominant and encephalitogenic epitopes, the rates of appearance of this peptide from the normal MBP-C1, normal MBP Cit_6, and Marburg MBP Cit_{18} were compared (Figure 3B). From visual

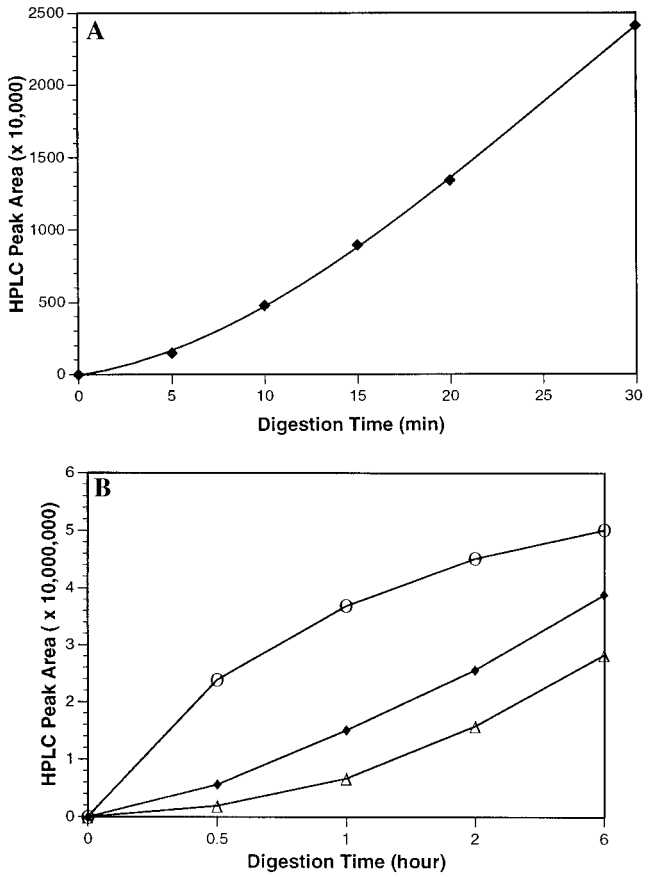

FIGURE 3: Generation of peptide 45−89. (A) The areas under the peak for MBP peptide 45−89 were summed and plotted against the time of digestion of Marburg MBP Cit_{18} from 0 to 30 min. (B) The areas under the peaks for MBP peptide 45−89 appearing during digestion by cathepsin D of normal MBP-C1 (△), normal MBP Cit_6 (♦), and Marburg MBP Cit_{18} (○) over 6 h were also plotted.

examination, it is clear that peptide 45−89 was released in order of decreasing citrulline content most rapidly from Marburg MBP Cit_{18}, normal MBP Cit_6, and normal MBP-C1.

A Cathepsin D Digestion of MBP-C1 and MBP-C8 Isomers from Normal and Chronic MS Brain Tissue. These chromatograms are shown in Figure 4. After digestion for 360 min, the chromatograms for MBP-C1 from normal brain were similar to that of MBP-C1 from chronic MS brain, neither of which contains citrulline (Figure 4A,B). MBP Cit_6 from normal brain was similar to MBP Cit_6 from chronic MS brain (Figure 4C,D). Both normal and MS MBP Cit_6 isomers contain 6 mol of citrulline per mole of protein. MBP peptide 45−89 from both MBP-C8 isomers sometimes chromatographed as a bifid peak and was refractory with respect to further degradation. These two peptides were separated by rechromatography on HPLC and purified. Their identification as MBP peptide 45−89 was confirmed by reactivity with mAb 845 D3, specific for MBP peptide 80−89, and by amino acid analyses (data not shown). Mass spectrometry of these two peaks gave molecular masses of 4878.05 ± 0.89 Da for the first peak and 4880.16 ± 0.22 Da for the second peak. The difference of 2 Da is most readily explained by deamidation of two glutamines or one glutamine and one asparagine (citrulline has not been detected in any of our MBP Cit_6 samples except from Marburg's disease in this part of the protein). MBP Cit_6

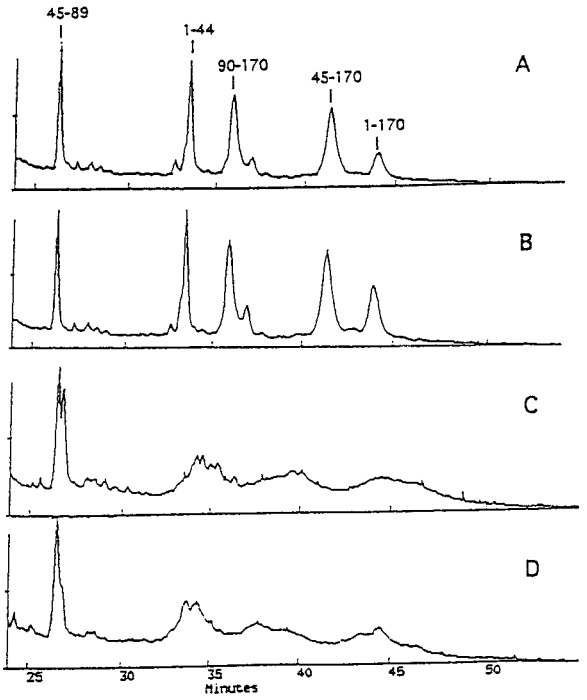

FIGURE 4: Comparative digestion of MBP-C1 and MBP Cit_6 from normal and chronic MS brain tissue by cathepsin D. Normal MBP-C1 (A), MS MBP-C1 (B), normal MBP Cit_6 (C), and MS MBP Cit_6 (D) were digested with cathepsin D for 6 h and separated by HPLC as described in the legend of Figure 1. The HPLC profile correlates with the presence of citrulline and is not different in MS and normal MBP isomers.

peptide 1−44 eluted as a number of small peaks between 33 and 34 min in the C8 samples. All reacted with mAb F23 which is specific for MBP peptide acetyl 1−9. This multiplex elution profile of MBP peptide 1−44 is related to extensive modifications reported in this region of MBP, such as the heterogeneous N-terminus (*23*) and the presence of two citrulline residues (*8*). Two of the peaks of MBP peptide 1−44 were further purified for mass spectrometry, from which molecular masses of 5003.56 ± 0.57 and 5004.51 ± 0.51 Da were obtained. The difference of 1 Da is consistent with the presence of a citrulline residue in this peptide.

Secondary Structure of Peptide 45−89. Because MBP peptide 45−89 derived from any of the MBP isomers that were examined appeared to be unusually stable in the presence of cathepsin D whereas some of the other MBP peptides were degraded, we determined its secondary structure by circular dichroism. Studies in a number of laboratories in a variety of solvent systems have failed to detect specific α-helical or *β*-structures in MBP (*24*−*28*). However, a specific secondary structure determined by immunochemical means (*29*) showed that antibody to bovine MBP peptide 43−88, the equivalent of human MBP peptide 45−89, reacted well with the peptide but showed little or no reaction with intact MBP. They concluded that MBP peptide 43−88 was shielded and must be internalized in a folded structure in MBP. Since this peptide contains an immunodominant epitope for myelin basic protein-like material in cerebrospinal fluid (*12*) and urine (*13*), release of this MBP peptide by cathepsin D digestion creates a source of this peptide for sensitization of T cells.

Circular dichroism of MBP peptide 45−89 released from both normal MBP-C1 and Marburg MBP Cit_{18} (Figure 5),

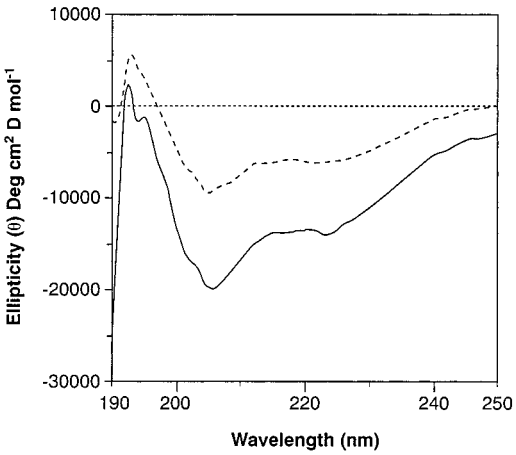

FIGURE 5: Circular dichroism spectra of MBP peptide 45−89 from normal MBP-C1 and Marburg MBP Cit_{18}. MBP peptide 45−89 from normal MBP-C1 (—) and Marburg MBP Cit_{18} (- - -) were dissolved in 87.5% 2-propanol for determinations.

dissolved in 87.5% 2-propanol to provide a lipid mimetic environment, revealed α-helical spectra in both peptides. This suggests that in a lipid rich environment, such as that found in myelin, the MBP peptide 45−89 should be α-helical. This specific secondary structure may protect the peptide from proteolytic degradation and may explain its unusual stability. Although the presence of four citrullinyl groups in Marburg MBP Cit_{18} peptide 45−89 decreases the molar ellipticity, the spectrum remains α-helical (Figure 5).

DISCUSSION

In studying the chemical nature of MBP isolated from victims of multiple sclerosis, we found that the level of the citrullinated form of MBP was elevated 2−3-fold in chronic MS (*9*). In a single case of fulminating MS (Marburg type), more than 90% of the MBP was the citrullinated form (*10*). Whereas the citrullinated MBP from chronic MS contained six or seven residues of citrulline per mole of protein, that from the Marburg case contained 18 residues of citrulline per mole. In a recent report about rheumatoid arthritis sera, citrulline was found to be an essential constituent of antigenic determinants present in filaggrin which were recognized by rheumatoid arthritis-specific autoantibodies (*30*, *31*). When considered with our MS data, these studies suggest that the conversion of arginine to citrulline in proteins by the enzyme peptidylarginine deiminase in both MS and rheumatoid arthritis represents an important part of the chemical pathology of both these diseases with major autoimmune components. Peptidylarginine deiminase, the enzyme which carries out the deimination of arginyl residues in proteins, has been mapped to chromosome 1p36 in our laboratory (unpublished data). In a genome-wide linkage study for rheumatoid arthritis, a chromosome 1 locus was identified as a susceptibility locus (*32*). In a Japanese study of rheumatoid arthritis patients, 1p36 was identified as a susceptibility locus, but was not correlated with peptidylarginine deiminase (*33*). The presence of citrulline-containing peptides in both these diseases and the possible identification of a common chromosomal susceptibility locus suggest that deimination of arginine increases its susceptibility to protease digestion of filaggrin in rheumatoid arthritis and to MBP in MS. Furthermore, detection of these peptides in body fluids

suggests that the presence of citrulline increases their stability. Thus deimination may be viewed as a first step in making available encephalitogenic peptides in MS and immunogenic peptides in rheumatoid arthritis.

MBP peptide 45−89, which contains the immunodominant epitope detected in body fluids of MS patients, was found to be unavailable to antibody in the intact protein but readily available as the isolated peptide (*29*). This suggests it is internalized in a folded structure in the intact MBP molecule and therefore not available to T or B cells. This view is in keeping with the report of 25% β-structure in bovine MBP in a combined FTIR and Raman study (*34*). Liberation of this peptide with cathepsin D makes it available to these cells. The presence of secondary structure in the liberated peptide helps to protect it from further enzymic digestion so that an immunodominant epitope can be carried out of the central nervous system where it can interact with cells of the immune system.

Although the argument that the loss of positive charge by the presence of citrulline in MBP may lead to polymerization (aggregation) cannot be ruled out totally, it is unlikely to be the explanation for the greater susceptibility of citrullinated MBP to degradation by cathepsin D. At pH 7.0, MBP has 31 positive charges (19 Arg and 12 Lys) and 11 negative charges (9 Asp and 2 Glu) for a net positive charge of +20. At pH 3.5 (the pH at which cathepsin D digestions were performed), the carboxyl groups of aspartic acid and glutamic acid with p*K* values of about 3.5 would only be 50% negatively charged. Therefore, C1 at pH 3.5 would have 31 positive charges but only 50% of the 11 negative charges for a net charge of +25. With six citrullinyl residues in MBP Cit_6 (or C8), the molecule would still have 19 positive charges. MBP Cit_{18} (Marburg's C8) would have 13 positive charges (12 Lys and 1 Arg) and 5.5 negative charges for a net positive charge of +7.5. Although there may be a greater tendency to aggregate in MBP Cit_{18}, it is unlikely at pH 3.5. It would definitely be a consideration at pH 7.0 where the net positive charge would be 2.0.

With this view in mind, we studied the degradation of several citrullinated MBP charge isomers and compared these with noncitrullinated MBP. The enzyme that was employed was cathepsin D, a myelin-associated enzyme which had been implicated previously in lesion production in MS (*15*−*17*). We were able to demonstrate that MBP Cit_6 isolated from normal human brain or from MS brain, which contained 6 mol of citrulline, was degraded more quickly than MBP-C1 which does not contain citrulline. MBP Cit_{18}, which contained 18 citrullinyl residues from the Marburg case, was degraded more rapidly than normal MBP-C1 or MBP Cit_6, demonstrating that the greater numbers of citrulline residues, the greater the susceptibility to enzyme digestion. Furthermore, the pattern of cleavage of MBP-C1-generated peptides from the expected cleavage sites at the two Phe−Phe linkages (44−45 and 89−90) was unaltered even after digestion for 6 h (Figure 1). More extensive degradation was observed when the citrullinated MBP Cit_6 from normal or chronic MS was digested. With the Marburg MBP Cit_{18}, the presence of more citrulline in the MBP molecule may have exposed minor cleavage sites in MBP to cathepsin D. MBP peptide 45−89, which contains the immunodominant epitope in CSF (*35*) and urine (*13*), survived cathepsin D digestion in samples of all MBP isomers that were tested. It is especially noteworthy that in the digest of Marburg MBP Cit_{18}, MBP peptide 45−89 appeared quickly, within 5 min of digestion, and survived through 360 min of digestion. The ability of this peptide to survive may be related to its role as an immunodominant peptide. For MBP-C1, this peptide does not appear until after 30 min. We postulate that the first step in generating this encephalitogenic peptide is the deimination of MBP by peptidylarginine deiminase. The more extensive the deimination, the more rapidly this peptide is generated. In fact, the rate of appearance of MBP peptide 45−89 was 35 times greater when the Marburg MBP Cit_{18} was digested than when MBP-C1 was digested.

A possible explanation for why Marburg MBP Cit_{18} was so much more susceptible to cathepsin D degradation may be related to the three-dimensional structure of citrullinated MBP obtained by electron microscopy for studying the folded structure of MBP, a method which does not require crystallization (*36*, *37*). A molecular resolution of about 20 Å could be achieved. This is 1 order of magnitude less sensitive than X-ray crystallography so that specific amino acid residues could not be identified, but it generated a folded structure model (Beniac, D. R., et al., submitted for publication). The model obtained for Marburg MBP Cit_{18} was larger and less compact than normal MBP-C1 or MBP Cit_6. This less compact structure of Marburg MBP Cit_{18} would be more accessible to protease digestion than the uncitrullinated MBP-C1 or the less citrullinated normal MBP Cit_6. These studies are continuing to improve resolution to a level which would permit identification of specific amino acid residues to determine if MBP peptide 45−89 resides in a protected structural environment.

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