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Research Article

Multiple sclerosis (MS) is an autoimmune disease in which myelin proteins have been implicated as autoantigens recognized by pathogenic autoreactive T cells. To study the relationship between human myelin basic protein (hMBP) and HLA alleles associated to MS susceptibility, such as DRB1*1501, the binding of synthetic peptides spanning the entire hMBP sequence to 10 purified HLA-DR molecules was determined. All the hMBP peptides tested showed binding affinity for at least one of the DR molecules analyzed, but three hMBP peptides, included in sequences 13-32, 84-103, and 144-163 were found capable of binding to three or more DR molecules. The hMBP peptide 84-103 was the most degenerate in binding, in that it bound to 9 out of 10 DR molecules tested. Interestingly, it bound with highest affinity to DRB1*1501 molecules. To correlate the binding pattern of hMBP peptides to HLA class II molecules with their recognition by T cells, 61 hMBP-specific T cell lines (TCL) were established from the peripheral blood of 20 MS patients, who were homozygous, heterozygous, or negative for DRB1*1501. Analysis of hMBP epitopes recognized by these TCL and their HLA restriction demonstrated a very good correlation between binding data and T cell proliferation to hMBP peptides. Although virtually all hMBP peptides tested could be recognized by at least one TCL from MS patients, three immunodominant T cell [...]

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Binding of Myelin Basic Protein Peptides to Human Histocompatibility Leukocyte Antigen Class II Molecules and Their Recognition by T Cells from Multiple Sclerosis Patients

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Abstract

Multiple sclerosis (MS) is an autoimmune disease in which myelin proteins have been implicated as autoantigens recognized by pathogenic autoreactive T cells. To study the relationship between human myelin basic protein (hMBP) and HLA alleles associated to MS susceptibility, such as DRB1*1501, the binding of synthetic peptides spanning the entire hMBP sequence to 10 purified HLA-DR molecules was determined. All the hMBP peptides tested showed binding affinity for at least one of the DR molecules analyzed, but three hMBP peptides, included in sequences 13–32, 84–103, and 144–163 were found capable of binding to three or more DR molecules. The hMBP peptide 84–103 was the most degenerate in binding, in that it bound to 9 out of 10 DR molecules tested. Interestingly, it bound with highest affinity to DRB1*1501 molecules. To correlate the binding pattern of hMBP peptides to HLA class II molecules with their recognition by T cells, 61 hMBP-specific T cell lines (TCL) were established from the peripheral blood of 20 MS patients, who were homozygous, heterozygous, or negative for DRB1*1501. Analysis of hMBP epitopes recognized by these TCL and their HLA restriction demonstrated a very good correlation between binding data and T cell proliferation to hMBP peptides. Although virtually all hMBP peptides tested could be recognized by at least one TCL from MS patients, three immunodominant T cell epitopes were apparent among the TCL examined, corresponding exactly to the hMBP peptides capable of binding to several DR molecules. No major difference could be detected in the recognition of immunodominant hMBP peptides by TCL from DRB1*1501 positive or negative MS patients. These results have implications for the role of hMBP as relevant autoantigen, and of DRB1*1501 as susceptibility allele in MS. (*J. Clin. Invest.* 1993. 91:616–628.) Key words: immunodominance • human histocompatibility

leukocyte antigen class II molecules • multiple sclerosis • myelin basic protein • T cell epitopes

Introduction

Multiple sclerosis (MS)¹ is a chronic, inflammatory disease of the human central nervous system (CNS) characterized by demyelination and by focal infiltrates of macrophages, plasma cells, and T cells in the CNS (1). Although the cause of MS is unknown, a T cell-dependent autoimmune process, involving autoreactive T cells specific for a myelin protein, has been postulated (reviewed in reference 2) and the demonstration of in vivo-activated T cells specific for human myelin basic protein (hMBP) in the blood of MS patients has implicated hMBP as a candidate autoantigen in the pathogenesis of MS (3).

The possible role of hMBP in MS has been further supported by the observation that experimental allergic encephalomyelitis (EAE), an animal model for MS, can be induced by immunization with MBP or MBP peptides in adjuvant (4, 5), as well as by passive transfer of MBP-specific CD4⁺ T cells (6–8). Encephalitogenic T cells in mice and rats are specific for immunodominant MBP epitopes presented by specific MHC class II molecules; for example, in B10.PL mice encephalitogenic T cells recognize the amino-terminal MBP peptide 1–9 presented by I-A^u molecules (9). The variable regions of the T cell receptor (TCR) in MBP-specific T cells have been found of limited heterogeneity, with a preferential expression of V β 8.2 and V α 2 or V α 4, both in mice and rats (10). Thus, in these species, MHC class II and TCR gene products associated to recognition of encephalitogenic MBP epitopes by T cells have been identified. Recently, however, it has been demonstrated that EAE can be induced by other autoantigens, such as proteolipid protein (11), raising the possibility that a multiplicity of myelin antigens may be capable of triggering autoimmune reactions resulting in the clinical manifestations characteristic of EAE.

A significant association between major histocompatibility complex (MHC) class II gene products and disease has been observed in MS patients. The higher concordance rate for MS in monozygotic compared to dizygotic twins indicates a genetically determined susceptibility to the disease (12). Because the frequency of the HLA class II allele DRB1*1501, and of the associated DQB1*0602, is significantly increased in Northern

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1. Abbreviations used in this paper: APC, antigen-presenting cells; EAE, experimental allergic encephalomyelitis; EDSS, expanded disability status scale; hMBP, human myelin basic protein; MS, multiple sclerosis; TCL, T cell line; TCR, T cell receptor.

European MS patients compared to controls, DRB1*1501, or gene(s) in linkage disequilibrium with it, could represent a susceptibility gene for MS (13, 14). Although the DRB1*1501 haplotype is associated with the highest relative risk of MS, other HLA class II alleles are overrepresented among certain ethnic groups of MS patients: DR4 in Southern Italians (15) and Arabs (16) and DR13 in Japanese (17) and Mexicans (18). In contrast to the well-established association between HLA genes and MS, the association of TCR genes, either in the germline (19, 20) or in the hMBP-specific peripheral T cell repertoire (21–25), with MS is still unclear.

To assess the possible role of hMBP as autoantigen in MS, several groups have studied the epitope specificity of hMBP-reactive T cells in MS patients and controls (26–32). Autoreactive T cells, mostly CD4⁺ and DR-restricted, specific for several hMBP peptides have been obtained from both MS patients and healthy controls. Although, collectively, major differences were not apparent in the T cell reactivity to hMBP between MS patients and controls, it has been suggested that recognition of immunodominant hMBP epitopes in individuals expressing an MS-associated HLA class II allele could be important in the pathogenesis of the disease. This would be expected if hMBP is the autoantigen in MS, and if the role of the MS-associated class II alleles is to present hMBP peptides to autoreactive T cells.

To test this hypothesis, the binding of a panel of hMBP peptides to several purified DR molecules, including those associated to increased frequency of MS, was determined. In addition, T cell lines (TCL) specific for hMBP were established from MS patients positive or negative for the MS-associated allele HLA-DRB1*1501.

As demonstrated in the present study, some hMBP peptides bind with highest affinity to HLA class II molecules encoded by alleles associated to increased frequency of MS, thus providing a possible molecular explanation for the association between class II genes and MS, and at the same time implicating the hMBP sequence 84–103 as a possible candidate for the autoantigenic determinant. The finding that this peptide binds, with lower affinity, to several other DR molecules, may also provide an explanation for the incomplete association between MS and class II genes.

However, the observation that hMBP-specific T cells from either DRB1*1501-positive or -negative MS patients recognize equally well this and other hMBP peptides cautions against a simplistic interpretation of the binding data, and illustrates the complexity and heterogeneity of T cell responses against hMBP.

Methods

Antigens. hMBP was prepared as described (33). Purity of hMBP preparations, as assessed by silver staining after gel electrophoresis, was routinely found to be > 90%. Peptides were synthesized on an Applied Biosystems Inc. (Foster City, CA) 430A peptide synthesizer. After removal of the α -amino-tert-butyloxycarbonyl protecting group, the phenylacetamidomethyl resin peptide was coupled with a fourfold excess of preformed symmetrical anhydride (hydroxybenzyltriazole esters for arginine, histidine, asparagine, and glutamine) for 1 h in dimethylformamide. For arginine, asparagine, glutamine, and histidine residues, the coupling step was repeated to obtain a high coupling efficiency. After synthesis was completed, the peptide was cleaved from the resin and the protecting groups removed by treatment with hydro-

gen fluoride in the presence of appropriate scavengers. The peptides were then purified by reversed phase high-performance liquid chromatography (HPLC). Peptide purity was substantiated by amino acid sequence and/or composition analysis. They were routinely > 95% pure after HPLC.

Affinity purification of DR molecules. The following Epstein-Barr virus (EBV)-transformed homozygous cell lines—LG2 (DRB1*0101), 3107 (DRB5*0101), MAT (DRB1*0301), PRIESS (DRB1*0404), BIN40 (DRB1*0404), SWEIG (DRB1*1101), PITOUT (DRB1*0701); or transfected fibroblasts—L466.1 (DRB1*1501), L416.3 (DRB5*0101), L242.5 (DRB1*1601), L255.1 (DRB5*0201)—were used as a source of HLA class II molecules. DR-transfected fibroblast were a kind gift of Dr. R. W. Karr (University of Iowa, Iowa City, IA). The HLA nomenclature is according to reference 34. Cell lines were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) or horse serum (Hazelton Biologics, Inc., Lenexa, KS). Cells were lysed at a concentration of 10⁸ cells/ml in 50 mM Tris-HCl, pH 8.5, containing 2% Renex, 150 mM NaCl, 5 mM EDTA, and 2 mM phenylmethyl sulfonyl fluoride (PMSF). The lysates were cleared of nuclei and debris by centrifugation at 10,000 g for 20 min.

DR molecules were purified essentially as described (35, 36) using the mAb LB3.1 (37) covalently coupled to protein A-Sepharose CL-4B. Aliquots of cell lysates equivalent to ~ 10 g of cells were passed sequentially through the following columns: Sepharose CL-4B (10 ml), protein A-Sepharose (5 ml), W6/32-protein A-Sepharose (10 ml), and LB3.1-protein A-Sepharose (15 ml) using a flow rate of 15 ml/h. The columns were washed with 10 column volumes of 10 mM Tris-HCl, pH 8.0, 0.1% Renex (5 ml/h); 2 column volumes of PBS, and 2 column volumes of PBS-1% octylglucoside. DR molecules were eluted from the LB3.1 column with 0.05 M diethylamine, in 0.15 M NaCl containing 1% octylglucoside (pH 11.5), immediately neutralized with 2 M glycine, pH 2.0, and concentrated by ultrafiltration through a Amicon YM-30 membrane (Amicon Corp., Danvers, MA). Protein content was evaluated by a BCA protein assay (Pierce Chemical Co., Rockford, IL) and confirmed by SDS-PAGE.

Binding of hMBP peptides to purified HLA class II molecules. Purified DR molecules (5–500 nM) were incubated with 5 nM ¹²⁵I-radiolabeled peptides for 48 h in PBS containing 5% dimethylsulfoxide (DMSO) in the presence of a protease inhibitor mixture. Purified peptides were iodinated using the chloramine-T method. The final concentrations of peptide inhibitors were: 1 mM PMSF, 1.3 mM 1.10 phenanthroline, 73 μ M pepstatin A, 8 mM EDTA, 6 mM N-ethylmaleimide, and 200 μ M N-Ap-tosyl-L-lysine chloromethyl ketone (TLCK). Final detergent concentration in the incubation mixture was 0.05% NP-40. Assays were performed at pH 7.0 with the exception of DRB1*0301, which was performed at pH 4.5, and DRB1*1601, which was performed at pH 5. The pH was adjusted as previously described (38). The DR-peptide complexes were separated from free peptides by gel filtration on Sephadex G50 columns as previously described (39, 40) or TSK2000 (7.8 mm $\times$ 15 cm) eluted at 1.2 ml/min in PBS, pH 6.5, containing 0.5% NP-40 and 0.1% NaN₃. Because the large size of the radiolabeled peptide used for the DRB1*1501 binding assay makes separation of bound from unbound peaks more difficult under these conditions, all DRB1*1501 assays were performed using a TSK2000 (7.8 mm $\times$ 30 cm) column eluted at 0.6 ml/min. Column eluates were passed through a model 170 radioisotope detector (Beckman Instruments, Inc., Palo Alto, CA), and radioactivity was plotted and integrated with a model 3396A integrator (Hewlett-Packard Co., Palo Alto, CA). The fraction of peptide bound was determined as previously described (40). The radiolabeled peptides used were: HA Y307-319 for DRB1*0101, DRB5*0201, and DRB1*1601; hMBP Y78-101 for DRB1*1501; MT 65 kD Y3-13 with Y7 substituted with F for DRB1*0301; a non-natural peptide with the sequence YARFQS-QTTLKQKT for DRB1*0401 and DRB1*0404; TT 830-843 for DRB5*0101, DRB1*1101, and DRB1*0701. In that the LB3.1 mAb used for DR purification is chain-specific, B1 molecules were not sepa-

rated from B3 molecules. The specificity of the binding assay for DRB1 molecules is obvious in the case of DRB1*0101 where no B3 chain is expressed, and has been demonstrated for DRB1*0301 (40), DRB1*0401, and DRB1*0404 (A. Sette, unpublished observations), DRB1*1101 (40), and DRB1*0701 (41). The problem of DRB chain specificity in assays for peptide binding to DRB1*1501, DRB5*0101, DRB1*1601, and DRB5*0201 is circumvented by the use of transfected fibroblasts. In preliminary experiments, each of the DR preparations was titrated in the presence of fixed amounts of radiolabeled peptides to determine the concentration of DR molecules necessary to bind 10–20% of the total radioactivity. All inhibition assays were performed using these DR concentrations and inhibitory peptides were typically tested at concentrations ranging from 120 $\mu\text{g/ml}$ to 1.2 ng/ml . The data were then plotted and the dose yielding 50% inhibition determined. Each peptide was tested in two to four independent experiments and results are presented as arithmetic mean of binding capacity expressed in nanomolar concentration.

Patients. 20 patients with clinically definite MS according to the Schumacher criteria (42) were included in this study. They were all free of immunosuppressive treatment at the time of blood sampling and had not received corticosteroids or adrenocorticotropin (ACTH) during the previous six months. Their disability status was assessed by the same neurologist (Dr. Kappos) and quantified by Kurtzke's expanded disability status scale (EDSS, 43). Disease activity at the time of first blood sampling was also assessed by the same rater according to the following criteria: Active disease (1) was defined as ongoing deterioration or relapse of the disease during the preceding 2 wk. Clinically inactive (0) were considered all patients without any deterioration or relapse during the past 12 mo. Patients who did not meet these criteria were considered probably active (2). In addition to the clinical assessment, magnetic resonance imaging (44, 45) was performed within 2 mo before the first blood sampling by a 2.0T Siemens Magnetom (Erlangen, FRG) according to the recommendations of the European Concerted Action on MS (46). Patients were divided according to the presence (1) or absence (0) of gadolinium enhancing lesions (45, 47). Informed consent was obtained from MS patients according to the requirements of the Declaration of Helsinki.

HLA typing. Typing for the polymorphism of HLA class II loci DRB1, DQA1, and DQB1 was performed using a standard technique for oligonucleotide typing of polymerase chain reaction-amplified DNA. Primers and oligonucleotides as well as hybridization and washing conditions were those recommended by the 11th International Histocompatibility Workshop (48). Nonradioactive labeling of oligonucleotides and chemiluminescence detection was performed according to Nevinny-Stickel et al. (49).

Establishment of MBP-specific TCL. Peripheral blood mononuclear cells (PBL) from MS patients, separated by Ficoll-Hypaque density gradient centrifugation, were resuspended in culture medium at 10^6 cells/ml and 200 μl was cultured in round-bottomed wells of microtiter plates (Nunc, Copenhagen, DK) together with 30 $\mu\text{g/ml}$ hMBP. Culture medium was RPMI 1640 (Gibco, Basel, CH) supplemented with 25 mM Hepes, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 50 $\mu\text{g/ml}$ gentamicin (Sigma Chemical Co., St. Louis, MO) and 10% heat-inactivated human AB positive serum. After 6 d of incubation at 37°C in a humidified atmosphere of 6% CO_2 in air, 100 μl of culture medium was replaced by fresh medium containing 20 U/ml human recombinant IL-2 (Hoffmann-La Roche, Basel). After 3–4 d the cultures were inspected microscopically for growth and positive cultures were split in complete medium with or without interleukin 2 (IL-2). After an additional 3–4 d, the latter cultures were washed twice and split into two adjacent microtiter wells. Irradiated (3,000 rad) autologous PBL (10^5 cells per well) were added to both wells and hMBP (30 $\mu\text{g/ml}$) to one of them. hMBP-specific proliferation was measured by tritiated thymidine incorporation for the last 6 h of a 3-d culture period. At day 15, hMBP-specific cell lines grown in medium containing IL-2 were restimulated with autologous irradiated PBL ($10^6/\text{ml}$) and hMBP (30 $\mu\text{g/ml}$) in medium containing IL-2. Thereafter, these cultures were restimulated in the same way every 7–10 d.

T cell proliferation assays. TCL were washed twice in RPMI and 5×10^4 cells were incubated with 10^5 irradiated autologous PBL per well. Alternatively, mitomycin c-treated homologous EBV-transformed B cells (2×10^4 cells per well) were used as antigen-presenting cells (APC). EBV-transformed B cells (EBV-B) were incubated ($10^7/\text{ml}$) with 50 $\mu\text{g/ml}$ mitomycin c (Sigma Chemical Co.) for 45 min at 37°C and then washed five times. Triplicate cultures were set up without antigen, with 30 $\mu\text{g/ml}$ hMBP or with 1 $\mu\text{g/ml}$ of hMBP synthetic peptides. Each TCL was always tested with the entire panel of 16 synthetic hMBP peptides. After 3 d of culture, 1 μCi per well tritiated thymidine (Amersham, Bucks, UK; 40 Ci/mmol) was added, the cells harvested 6 h later and thymidine incorporation measured by scintillation spectrometry.

Determination of HLA restriction. Monoclonal antibodies recognizing monomorphic determinants of DR (D1-12), DQ1 (BT3/4), DQ2 (XIII358/4), DQ3 (XIV466), and DP (B7/21) molecules were a gift from Dr. R. S. Accolla, University of Verona, Italy (50–52). APC were incubated with monoclonal antibodies (final dilution of ascitic fluid 1:1,000) for 2 h before addition of T cells and antigen. Cultures were then carried out as described above. A TCL was considered restricted by a given HLA class II isotype when inhibition by the corresponding monoclonal antibody was > 50% of the control response. In most cases, inhibition was between 80% and 100%. The HLA-DR allele restricting the response was determined by stimulating TCL with a panel of homozygous EBV-B cells obtained from the European Collection of Animal Cell Cultures (Porton, UK). Mitomycin c-treated EBV-B cells were pulsed with antigen for 4 h, then washed twice before addition of TCL.

Surface phenotype of T cell lines. The CD4 and CD8 phenotype of TCL was determined by cytofluorographic analysis. TCL were stained with anti-Leu-3 fluorescein isothiocyanate (FITC) (CD4) plus anti-Leu-8 PE (CD8), (Simultest, Becton, Dickinson & Co., Mountain View, CA) and analyzed by a FACScan flow cytometer.

Results

Binding of hMBP peptides to HLA class II molecules. We wished to examine the correlation between DR molecules associated with MS and their capacity to bind hMBP peptides. In addition, we wished to compare the capacity of hMBP peptides to bind to DR molecules with their antigenicity in vitro for hMBP-specific TCL derived from MS patients. Therefore, a panel of overlapping peptides spanning through the entire hMBP molecule was synthesized and tested for binding to several different HLA class II molecules (Tables I and II). The binding affinity of hMBP peptides to six different DR molecules is presented in Table I. As expected, different hMBP peptides bind, with different affinities, to distinct DR molecules. For example, three nonoverlapping peptides (84–103, 134–153, 153–170) bound with high affinity to DRB1*0101, whereas no high-affinity binders were detected for DRB1*0301 molecules. Overall, as documented in Tables I and II, 8/16 (50%) hMBP peptides bound with high or medium affinity (K_D 5–500 nM) to at least one DR molecule. Similar results were obtained when the binding capacity of hMBP peptides to different DR2 allelic products and isotypes was measured (Table II). Although all hMBP peptides examined showed some degree of binding affinity for at least one of the DR molecules tested, the three peptides corresponding to sequences 13–32, 84–103, and 144–163 were capable of binding with relatively high affinity (K_D < 500 nM) to several HLA class II molecules. This degenerate binding capacity was particularly evident for peptide 84–103, able to bind to 8 out of 10 HLA class II molecules tested with a K_D of 100 nM or less. Very high affinities were measured in the binding of hMBP peptide 84–103 to DR

Table I. Binding of hMBP Peptides to Different DRB1 Molecules

Sequence	Position	DRB1*0101	DRB1*0301	DRB1*0401	DRB1*0404	DRB1*1101	DRB1*0701
<i>nM</i>							
Ac-ASQKRPSQRHGSKYLATAST	Ac-1-20	—	—	5,000	—	—	2,273
KYLATASTMDHARHGFLPRH	13-32	13,000	—	100	87	5,000	157
HARHGFLPRHRDTGILDSIG	23-42	—	—	—	—	4,000	—
RDTGILDSIGRFFGGDRGAP	33-52	—	2,153	—	—	169	—
RFFGGDRGAPKRGSGKDSHH	43-62	—	—	5,000	—	—	—
GSGKDSHHPARTAHYGSLPQ	55-74	—	—	—	—	—	—
RTAHYGSLPQKSHGRTQDEN	65-84	—	—	—	—	3,333	—
QKSHGRTQDENPVVHFFKNI	74-93	1,667	—	900	1,515	—	12,500
NPVVHFFKNIVTPRTPPPSQ	84-103	13	—	7	36	17	66
VTPRTPPPSQGKGRGLSLR	94-113	10,000	—	5,625	16,667	10,000	—
GKGRGLSLSRFSWGAEGQRP	104-123	833	—	726	2,632	1,667	4,167
FSWGAEGQRPFGYGGGRASD	114-133	—	—	2,045	—	—	—
GFGYGGGRASDYKSAHKGFKG	124-143	13,000	—	15,000	—	139	—
YKSAHKGFKGVDAQGTLSKI	134-153	70	—	5,000	—	2,000	107
VDAQGTLSKIFKLGGRDSRS	144-163	25	4,545	9,000	314	18	1,136
IFKLGGRDSRSGSPMARR	153-170	25	—	—	—	10,000	—

Dash indicates binding > 20,000 nM.

molecules associated with increased MS susceptibility (DRB1*1501 and DRB1*0401). Interestingly, this peptide could also bind, although with lower affinities, to DR2 molecules encoded by alleles, such as DRB1*1601 and DRB5*0201, not associated with increased frequency of MS. Conversely, peptide 13-32 did not bind to DRB1*1501 molecules and 144-163 bound with very low affinity.

HLA DR and DQ alleles of MS patients. The salient clinical features of the MS patients analyzed are summarized in Table III. The HLA DR and DQ alleles of MS patients from whom hMBP-specific TCL were established are shown in Table IV.

The allele DRB1*1501 (formerly denominated DR2 Dw2), in linkage disequilibrium with DQB1*0602, occurs with significantly increased frequency in MS patients of Northern European ancestry (14). Among the 20 MS patients we have studied, 3 were DRB1*1501 homozygous, 6 heterozygous, and 11 negative. Therefore, the DRB1*1501 allele was present in 45% of the MS patients we analyzed, in agreement with other studies of HLA-MS association (13, 14).

Establishment of hMBP-specific TCL from MS patients. hMBP-specific TCL were obtained from 20 out of 28 (71%) MS patients tested. Out of the 61 TCL recognizing at least one

Table II. Binding of hMBP Peptides to Different DR2 Alleles and Isotypes

Sequence	Position	DRB1*1501	DRB5*0101	DRB1*1601	DRB5*0201
<i>nM</i>					
Ac-ASQKRPSQRHGSKYLATAST	Ac-1-20	—	—	545	9,100
KYLATASTMDHARHGFLPRH	13-32	—	10,000	—	4,200
HARHGFLPRHRDTGILDSIG	23-42	—	—	—	—
RDTGILDSIGRFFGGDRGAP	33-52	11,000	—	—	—
RFFGGDRGAPKRGSGKDSHH	43-62	7,300	10,000	—	—
GSGKDSHHPARTAHYGSLPQ	55-74	6,400	—	—	—
RTAHYGSLPQKSHGRTQDEN	65-84	2,900	—	—	—
QKSHGRTQDENPVVHFFKNI	74-93	43	1,850	341	125
NPVVHFFKNIVTPRTPPPSQ	84-103	5	156	21	98
VTPRTPPPSQGKGRGLSLR	94-113	—	—	—	—
GKGRGLSLSRFSWGAEGQRP	104-123	8,600	6,600	2,100	6,200
FSWGAEGQRPFGYGGGRASD	114-133	3,800	—	—	—
GFGYGGGRASDYKSAHKGFKG	124-143	2,000	136	—	16,000
YKSAHKGFKGVDAQGTLSKI	134-153	—	1,300	5,500	4,100
VDAQGTLSKIFKLGGRDSRS	144-163	7,700	6,600	388	1,500
IFKLGGRDSRSGSPMARR	153-170	1,600	3,300	15,000	4,700

Dash indicates binding > 20,000 nM.

Table III. Clinical Characteristics of MS Patients Included in The Study

Patient	Age	Sex	Disease duration	Disease course*	EDSS†	Disease activity	
						Gd enhancement‡	Clinical assessment
	yr		yr				
BV	23	F	2	2	4.0	0	0
BC	44	F	11	3	6.0	0	1
SE	38	F	17	4	6.0	1	1
BJ	66	M	41	4	6.0	0	2
OI	55	F	11	3	3.5	0	0
SJ	43	M	9	2	3.0	0	0
HU	44	F	1	1	1.5	1	0
TB	57	M	18	2	2.5	0	0
GA	39	F	12	2	3.5	0	0
LA	63	F	39	2	2.5	0	0
SI	54	F	7	4	2.5	0	0
KH	48	M	16	4	6.0	0	1
DV	45	F	1	2	2.0	1	0
SP	55	M	21	3	4.5	0	2
NA	35	F	7	4	4.0	1	2
SM	48	F	20	2	5.5	0	0
DM	37	M	17	1	1.5	0	0
SV	40	F	12	3	5.0	1	1
WB	58	F	8	4	6.0	1	1
MG	55	F	1	2	3.5	1	2

* Disease course: (1) relapsing-remitting with complete remissions, (2) relapsing-remitting with residual neurological deficit, (3) relapsing with progression between relapses, (4) secondary chronic progressive (disease initiated as relapsing-remitting and continued as chronic progressive).

† Disability is shown in grades of Kurtzke's EDSS. ‡ Disease activity evaluated by Gadolinium (Gd) – enhanced MRI: (1) enhancing lesions, (0) no enhancing lesions. || Disease activity evaluated by clinical assessment: (1) active, (2) probably active, (0) inactive (see text).

of the hMBP peptides tested, 26 (43%) were isolated from DRB1*1501-positive MS patients and 35 (57%) from DRB1*1501-negative MS patients (Table IV). A TCL was considered to be hMBP-specific when the stimulation index in the presence of 30 µg/ml hMBP was > 2 in at least two independent proliferation assays. The T cell proliferative response to MBP was very variable among different TCL, with stimulation indexes ranging from 2 to 1,100 (Table IV). In general, TCL were stable and maintained a similar level of proliferation to hMBP over a period of several months. No relationship was observed between expression of the allele DRB1*1501 by the MS patient and degree of responsiveness to hMBP in individual TCL. 15 DR-restricted TCL specific for hMBP peptides were tested for surface expression of CD4 and CD8 molecules: 5 TCL were mostly composed (> 70%) of CD4⁺ T cells, whereas 10 TCL contained, in addition to single-positive CD4⁺ cells, a substantial proportion (46–92%) of double-positive CD4⁺/CD8⁺ T cells (data not shown).

The majority of hMBP-specific TCL from MS patients are restricted by DR alleles. The HLA class II molecule presenting the hMBP peptides to hMBP-specific T cells was characterized, in terms of isotype, for 45 TCL (Table IV). The HLA class II isotype restricting the anti-hMBP T cell response was assessed by inhibition of T cell proliferation by anti-DR, anti-DQ, and anti-DP monoclonal antibodies specific for HLA class II monomorphic determinants. Representative examples of blocking by anti-class II monoclonal antibodies are shown in Fig. 1. All

the 45 TCL tested were found to be restricted by a single HLA class II isotype. The majority of hMBP-specific TCL (38/45, 85%) were DR restricted. Few TCL (3/45, 6%) recognized hMBP peptides presented by DQ molecules. Interestingly, DQ-restricted TCL were only obtained from MS patients homozygous for DQA1*0102/DQB1*0602. Some TCL (4/45, 9%) were restricted by DP molecules.

hMBP epitopes recognized by hMBP-specific TCL from MS patients. 61 hMBP-specific TCL established from 20 MS patients responded to one or more of the hMBP peptides tested. hMBP epitope(s) were recognized by ~ 75% of the hMBP-specific TCL tested. Most T cell lines were specific for a single sequence of hMBP, but in some cases T cell proliferation to nonoverlapping hMBP peptides was observed. The proliferative response to hMBP and to synthetic hMBP peptides of four representative TCL is shown in Fig. 2. The hMBP T cell epitopes recognized by all TCL tested, as well as the degree of responsiveness expressed as stimulation index, is presented in Table IV. T cell proliferation to hMBP and to hMBP peptides was dose dependent, and was usually induced by lower molar concentrations of the appropriate synthetic peptide, as compared to hMBP (Fig. 3).

The 61 hMBP-specific TCL tested responded, overall, to 78 hMBP peptides. The hMBP peptides recognized by TCL, irrespective of their HLA restriction, are presented in Fig. 4. Of the 16 hMBP peptides tested, 14 were recognized by at least one hMBP-specific TCL. Only two hMBP peptides, 23–42 and 74–

Table IV. Epitope Specificity and HLA Class II Restriction of hMBP-specific TCL from MS Patients

Patient	DRB1*	DRB3/4	DQA1*	DQB1*	Line no.	hMBP (SI)	Peptide	SI	Restriction
BV	1302, 07	w52, w53	0102, 0201	0604, 0201	11	553	84-103	685	DRB1*1302
BC	1501, 07	w53	0102, 0201	0602, 0201	27	65	84-103	69	DR
					41	133	84-103	210	DR
					44	214	43-62	64	DR
SE	1051, —		0102, —	0602, —	3	114	55-74	176	
BJ	0101, 0401		0101, 0301	0501, 0302	3	3	144-163	3	
					9	5	84-103	15	
					10	299	144-163	294	DRB1*0101
							153-170	75	
OI	1101, —	w52	0501, —	0301, —	15	14	144-163	11	
					19	170	33-52	73	
							84-103	25	
							144-163	82	
SJ	1501, —		0102, —	0602, —	1	434	13-32	455	DQ
					28	9	104-123	4	DQ
HU	1501, 0401	w53	0102, 0301	0602, 0302	2	5	13-32	3	
					9	10	13-32	6	
TB	1501, —		0102, —	0602, —	2	2	13-32	2	DQ
					7	2	84-103	2	DR
					9	3	84-103	3	DR
					12	7	104-123	3	
GA	1101, 0801	w52	0401, 0501	0301, 0402	2	500	144-163	560	DR
					7	120	33-52	20	DR
					10	180	13-32	12	DR
					22	3	124-143	10	DR
LA	0404, 1101	w52, w53	0301, 0501	0302, 0301	1	135	84-103	65	DR
					8	12	84-103	12	DRB1*1101
							144-163	14	
					9	90	84-103	10	DR
					13	133	84-103	150	
					14	6	13-32	3	DR
					21	279	84-103	350	DRB1*1101
					33	140	144-163	190	DR
SI	1101, 1001	w52	0101, 0501	0501, 0301	2	319	33-52	27	DRB1*1101
					4	30	84-103	6	DRB1*1101
					6	13	124-143	25	DR
					9	110	84-103	17	DR
							134-153	120	
					14	98	84-103	33	DR
							134-153	79	
					17	15	84-103	10	
KH	1301, 07	w52, w53	0103, 0401	0201, 0402	1	12	84-103	17	DP
					20	60	144-163	37	DR
					21	18	144-163	22	DR
DV	0301, 0803	w52	0501, 0301	0201, 0601	2	80	1-20	20	DP
					24	330	65-84	350	
					26	1100	124-143	65	DR
					29	110	94-113	55	
SP	1501, 1301	w52	0103, 0102	0602, 0603	9	980	144-163	800	DRB1*1301
					10	230	144-163	15	DRB1*1301
					38	19	33-52	10	DRB1*1501 (DRB5*0101)
					39	10	134-153	6	DR
							153-170	9	
NA	1501, 1301	w52	0102, 0103	0602, 0603	15	110	84-103	70	DR
							144-163	120	
					18	12	144-163	38	DP
SM	1501, 1101	w52	0102, 0501	0602, 0301	3	40	13-32	76	

Table IV. (Continued)

Patient	DRB1*	DRB3/4	DQA1*	DQB1*	Line no.	hMBP (SI)	Peptide	SI	Restriction
DM	1501, 0401	w53	0102, 0301	0501, 0302	9	90	84-103	12	DRB1*1501 (DRB5*0101)
							144-163	22	
					11	220	84-103	80	DR
							144-163	105	
					17	42	84-103	40	DR
							144-163	51	
					30	102	1-20	188	DP
					35	38	84-103	5	DR
							134-153	33	
							144-163	7	
SV	0401, —	w52, w53	0301, —	0302, —	42	51	104-123	9	DRB1*1501 (DRB5*0101)
					58	65	84-103	17	DRB1*1501 (DRB5*0101)
							144-163	25	
							65-84	3	
WB	01, 1301/2	w52	0101, 0501	0501, 0603	14	13	84-103	130	
					17	120	84-103	130	
							144-163	150	
					32	70	84-103	40	DR
MG	1302, 08	w52	0401, 0102	0402, 0604			144-163	20	
					42	40	114-133	90	DR
							124-143	70	
					7	120	124-143	8	

Dash indicates homozygosity. SI, stimulation index.

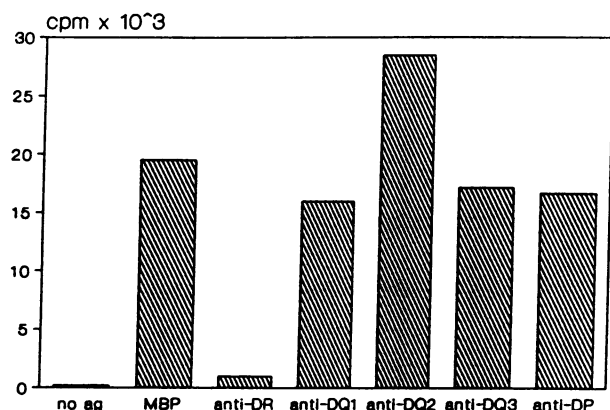
93, failed to induce a proliferative response in any of the hMBP-specific TCL examined. Therefore, practically all T cell epitopes of hMBP can be recognized by T cells from MS patients. However, two hMBP epitopes are clearly immunodominant. One is included in the hMBP sequence 84-103, inducing 25/78 (32%) peptide-specific T cell responses. This hMBP region appears to contain a nested set of overlapping epitopes (53), in that the truncated sequence 87-99 was recognized by only 30% of the TCL specific for 84-103 (not shown). A second immunodominant epitope, comprising 20/78 (25%) peptide-specific T cell responses, is located in the hMBP sequence 144-163. In addition, a third, less dominant T cell epitope, inducing 7/78 (9%) peptide-specific responses, is present in the hMBP sequence 13-32.

*Immunodominant hMBP epitopes do not differ in TCL from DRB1*1501 positive or negative MS patients.* Next, we wished to test whether a skewing in the immunodominant hMBP epitopes recognized was present in TCL derived from MS patients expressing DRB1*1501, as compared to patients not expressing it. Thus, TCL were subdivided according to the presence or absence of this allele in the MS patient blood donors. 34 hMBP peptides were recognized by the 26 TCL derived from nine DRB1*1501-positive MS patients, whereas 44 hMBP peptides were recognized by the 35 TCL obtained from the 11 MS patients not expressing DRB1*1501 (Table IV). Results in Fig. 5, presented as percent hMBP peptides recognized by TCL in the two subsets of MS patients, demonstrate that recognition of the two immunodominant epitopes included in the hMBP sequence 84-103 and 144-163 does not differ in TCL derived from DRB1*1501-positive or -negative patients. As to the third dominant epitope, defined by the hMBP peptide 13-32, five out of seven TCL were derived from

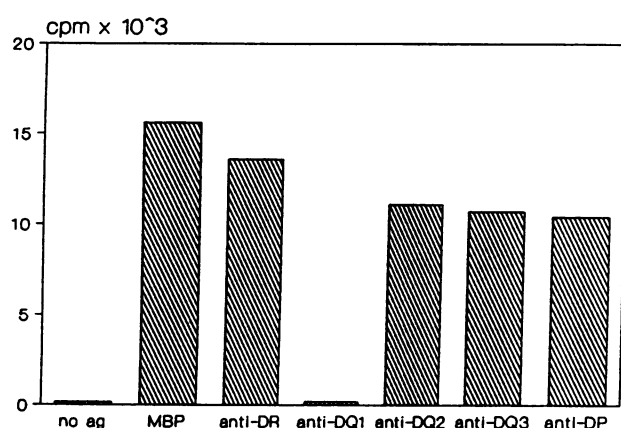
DRB1*1501-positive patients. However, three out of the five TCL from these MS patients, when tested for HLA class II isotype restriction, were found to be DQ restricted (Table IV, Fig. 1). This indicates that the hMBP peptide 13-32 can be recognized in DRB1*1501/DQB1*0602 MS patients complexed to either DR or DQ molecules, thus accounting for the increased frequency of responding TCL.

*HLA class II molecules presenting hMBP epitopes to hMBP-specific TCL from DRB1*1501 heterozygous or homozygous MS patients.* For some hMBP-specific TCL the HLA class II molecule presenting the hMBP peptide was determined by stimulating T cells with a panel of homozygous EBV-B cells. An example is shown in Table V. In this case, the DR-restricted TCL no. 9 from patient DM (DRB1*1501/DRB1*0401) was only restimulated by hMBP presented by EBV-B cells homozygous for DRB1*1501 or for the highly homologous allele DRB1*1502. These results confirm the DR restriction obtained by monoclonal antibody blocking. Since it was not determined whether the HLA molecule presenting the hMBP peptide is encoded by DRB1 or DRB5 genes, this TCL is restricted by either DRB1*1501 or DRB5*0101 class II molecules, as both molecules can present hMBP peptides to TCL (31, 54). In this assay the whole hMBP protein was used as antigen, indicating that both dominant hMBP epitopes (84-103 and 144-163) recognized by this TCL are restricted by DRB1*1501 or DRB5*0101 molecules. This was confirmed by presentation of the appropriate synthetic peptides by homozygous EBV-B cells (not shown).

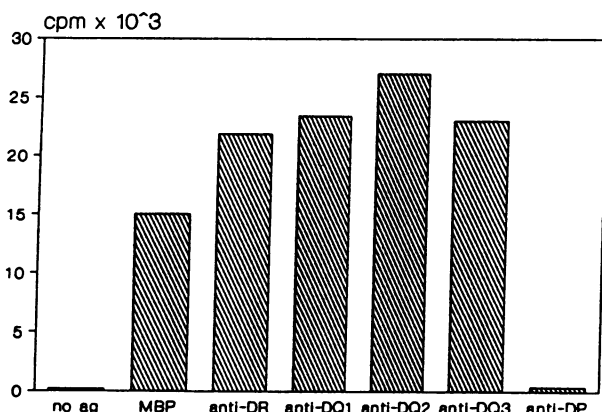
The DRB1 allele presenting hMBP peptides was determined by homozygous EBV-B cell-induced T cell proliferation for 12 TCL from DR-heterozygous patients (Table VI). In addition, five TCL from homozygous MS patients for which



NA line 15



SJ line 1



NA line 18

Figure 1. Characterization of HLA class II isotypic molecules presenting naturally processed hMBP peptides to hMBP-specific TCL. The HLA class II isotype restricting the anti-hMBP T cell response was assessed by inhibition of T cell proliferation by anti-DR, -DQ, and -DP monoclonal antibodies specific for HLA class II monomorphic determinants, as detailed in Methods. A description of these TCL is included in Table IV.

class II isotype restriction by antibody blocking was available, are also included. These results demonstrate that the dominant epitopes 84–103 and 144–163 can be presented by different class II molecules, as expected from their degenerate binding

capacity (Tables I and II). In particular, the peptide 84–103 can be presented to TCL by DRB1*1302, DRB1*1101, and DRB1*1501 (or DRB5*0101) molecules. The peptide 144–163 can be presented by at least four DR molecules: DRB1*0101, DRB1*1101, DRB1*1301, and DRB1*1501 (or DRB5*0101), further demonstrating the functional relevance of binding data.

These results highlight three points relevant to recognition of hMBP epitopes by TCL from DRB1*1501-positive MS patients. First, APC from MS patients carrying the DRB1*1501 haplotype can present to TCL at least six different hMBP peptides. Second, these peptides are not all presented by DRB1*1501 (or DRB5*0101) molecules. Third, in some cases the same peptide, e.g., 104–123, can be presented by either DR or DQ molecules.

Discussion

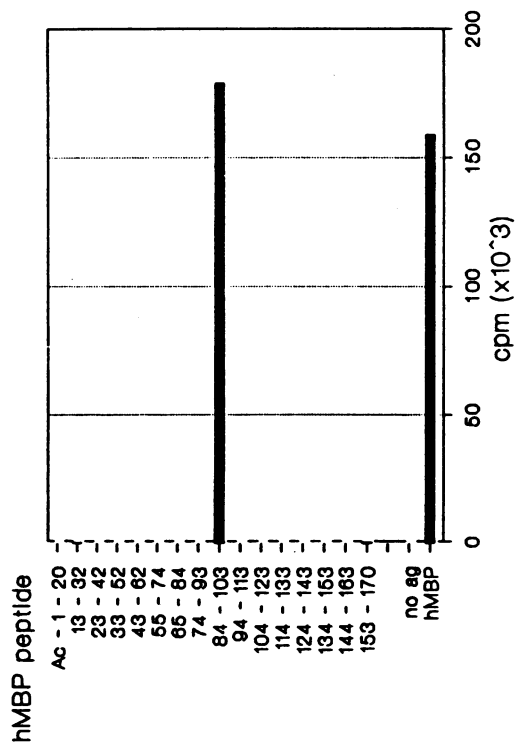
Based on experimental models of MS, hMBP is considered a likely candidate for the self antigen recognized by autoreactive, pathogenic human T cells capable of mediating MS (2, 55).

In the present study we have systematically analyzed the binding of overlapping peptides encompassing the entire hMBP molecule to several HLA class II molecules, and correlated their binding capacity to the recognition of hMBP peptides by HLA class II-restricted TCL. In particular, we have focused on the binding of hMBP peptides to HLA class II molecules associated or not to increased susceptibility to MS, and on the analysis of hMBP-specific TCL from MS patients expressing or not these susceptibility alleles. Although the emerging pattern is complex, some points appear to be quite clear.

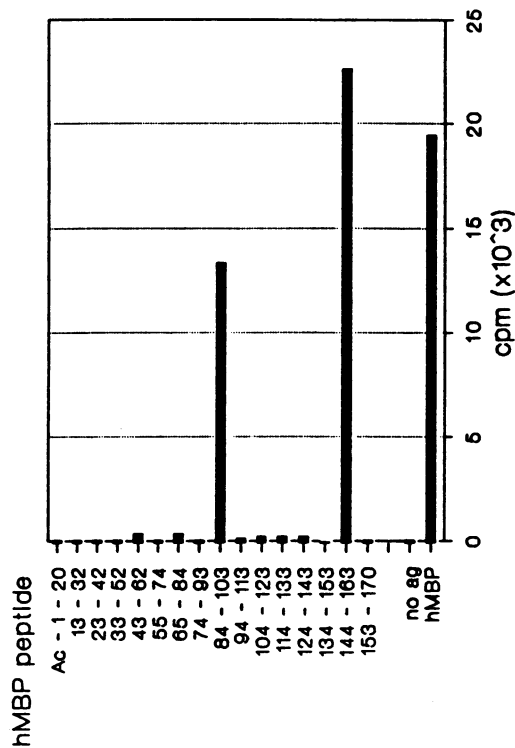
Several hMBP peptides bind with high affinity to one or two of the HLA class II molecules tested. In addition, three hMBP determinants, included in sequences 13–32, 84–103, and 144–163 exhibit degenerate binding, since they are able to form complexes with several of the HLA class II molecules examined. Degenerate binding of peptides to HLA class II molecules is not unprecedented because it has been described for peptides derived from malaria circumsporozoite protein (56), tetanus toxoid (57), and influenza hemagglutinin (41, 58, 59). Analysis of a large unbiased sample of naturally occurring sequences has revealed that degenerate binding is detectable only for a minority of peptides capable of binding to HLA class II molecules (39), and subsequent studies have illustrated that degenerate DR binding is characteristic of peptides capable of binding with high affinity to at least one DR molecule (59). To our knowledge, the present study shows the first example of degenerate binding to HLA class II molecules of peptides derived from a candidate autoantigen possibly involved in a human autoimmune disease.

A striking parallel exists between binding of hMBP peptides to HLA class II molecules and hMBP epitopes recognized by hMBP-specific TCL from MS patients. First, the peripheral T cell repertoire of MS patients, as predicted from binding data, includes T cells able to recognize almost any hMBP peptide. Second, the three hMBP peptides capable of degenerate binding (13–32, 84–103, and 144–163) correspond precisely to the three immunodominant epitopes we have identified in hMBP-specific T cell lines from MS patients. These results confirm previous studies indicating the immunodominance of hMBP peptides 84–103 and 144–163 (27, 29–32), and suggesting the existence of a third T cell epitope in the amino-terminal region

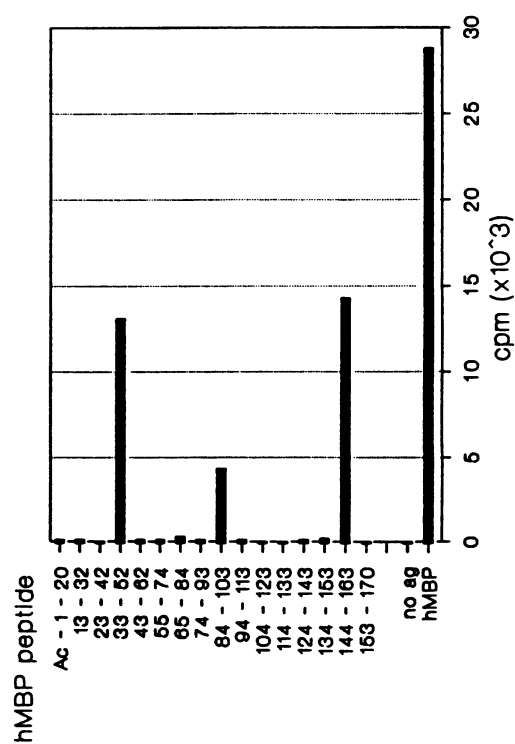
Line BV no. 11



Line NA no. 15



Line OI no. 19



Line LA no. 33

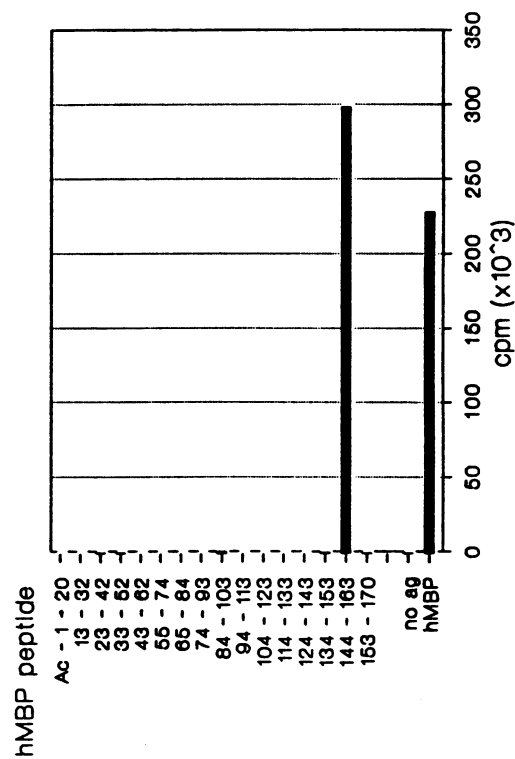


Figure 2. Proliferative response to hMBP and to synthetic hMBP peptides by four TCL (5×10^4 cells per well) and irradiated autologous PBL (10^5 cells per well) were cultured without antigen, with $30 \mu\text{g/ml}$ hMBP or with $1 \mu\text{g/ml}$ of the indicated synthetic peptides. After 3 d of culture $1 \mu\text{Ci}$ per well tritiated thymidine was added, the cells harvested 6 h later and [^3H]thymidine incorporation measured by scintillation spectrometry. Results are expressed as arithmetic mean of counts per minute (cpm) from triplicate cultures.

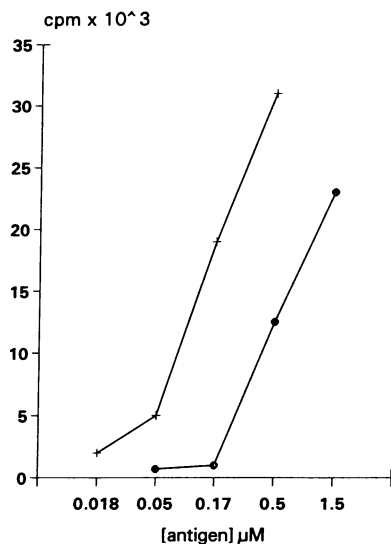


Figure 3. Dose-dependent T cell proliferation to hMBP and to the hMBP peptide 144–163. The TCL no. 10 from patient BJ (5×10^4 cells per well) was cultured with the indicated concentrations of hMBP (●) or hMBP peptide 144–163 (+) in the presence of mitomycin c-treated autologous EBV-transformed B cells (2×10^4 per well). Results are expressed as in Fig. 2.

of hMBP (30). In particular, the immunodominant hMBP epitopes corresponding to sequences 84–103 and 144–163 were previously identified, using overlapping hMBP peptides, by Ota et al. (29). Pette et al. (31) assigned at least three independent T cell epitopes within the thrombic hMBP fragment 131–170, and defined a fourth one using the synthetic peptide 80–99. Martin et al. (30) identified two immunodominant regions in the hMBP sequences 87–106 and 154–172. In addition, Martin et al. (53) have shown that the dominant peptide corresponding to the sequence 84–103 contains a nested set of epitopes presented to T cells by different HLA class II molecules, thereby implying degenerate binding of this hMBP sequence.

Immunodominance of T cell epitopes is certainly influenced by many mechanisms (60), but the present data indicate that, at the population level, degeneracy of binding plays an important role in determining immunodominance of selected T cell epitopes. The immunodominant T cell epitope included in the hMBP sequence 84–103 corresponds to the

peptide expressing the most degenerate binding pattern, since it binds to 8 out of the 10 DR molecules tested. In particular, this peptide binds with highest affinity, in the low nM range, to class II molecules encoded by DRB1*1501 and DRB1*0401 alleles. Because these alleles are associated to increased frequency of MS in Northern (14) and Southern (15) European populations, respectively, the results would suggest that high affinity binding of this hMBP peptide may be relevant to the pathogenesis of MS. Conversely, the other major immunodominant peptide, 144–163, binds very weakly to DRB1*1501 and DRB1*0401 molecules.

A point of debate is the binding affinity of autoantigenic peptides for the class II molecules associated to disease susceptibility. According to one theory, autoreactive pathogenic T cell clones could recognize low affinity binders, and for this reason have escaped thymic deletion during negative selection (61). The opposite view considers autoantigenic peptides as high affinity binders, and therefore highly antigenic. When events such as molecular mimicry (62) or regulatory imbalances (63) lead to breakdown of tolerance, their antigenic potential can be expressed. The hMBP peptide centered on the sequence 84–103 is a high-affinity binder to the DR molecules associated with increased frequency of MS. In analogy with binding of MBP peptides to mouse class II molecules (64), this result would suggest that high affinity binding may be necessary for a self peptide to become a dominant determinant recognized by autoreactive T cells.

Considering the binding data, and especially degeneracy of binding, it is therefore perhaps not surprising to find that the hMBP peptides 84–103 and 144–163 represent dominant epitopes recognized by about 30% of hMBP-specific TCL. In that only 84–103 binds with high affinity to DR molecules associated with increased frequency of MS, it would be tempting to infer that this epitope may be relevant to disease induction and/or progression. However, both immunodominant hMBP epitopes are recognized with the same frequency by TCL from DRB1*1501-positive or -negative MS patients. Moreover, in the three MS patients homozygous for DRB1*1501 many hMBP peptides are recognized by TCL in addition to 84–103, and in at least three of these TCL the response is restricted by

hMBP peptide

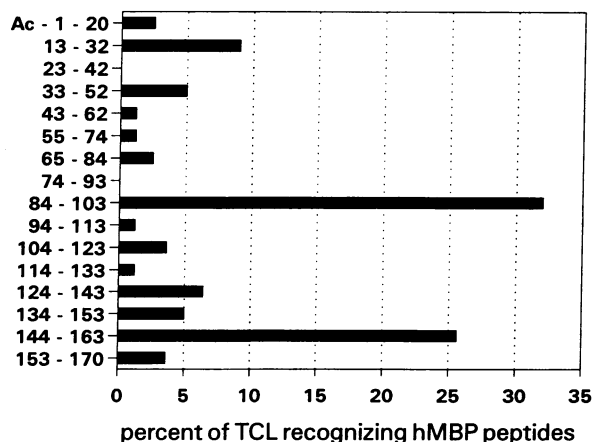


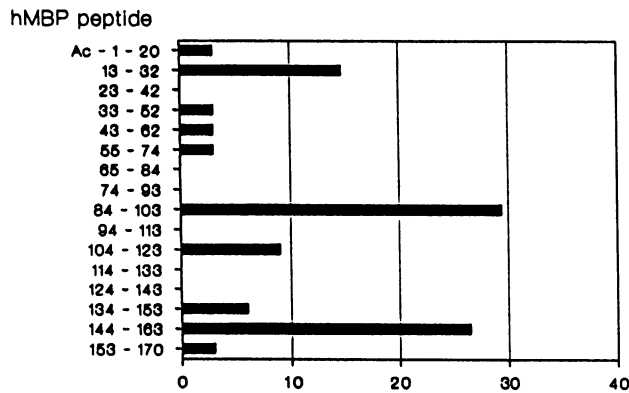
Figure 4. hMBP epitopes recognized by hMBP-specific TCL from MS patients. Results are expressed as percentage of TCL responding to a given hMBP peptide ($n = 78$).

Table V. HLA Class II Restriction of TCL DM No. 9

EBV-B cells	DRB1*	DRB3/4	No antigen	hMBP
<i>cpm</i>				
Autologous	1501, 0401	w53	3,557	31,593
MGAR	1501, —		1,410	27,165
E4181324	1502, —		582	16,281
KAS011	1601, —		351	575
RML	1602, —		3,155	2,340
PRIESS	0401, —	w53	138	83
PE117	0404, —	w53	255	159
BSM	0401, —	w53	351	443
MOU	0701, —	w53	1,140	577

The indicated EBV-transformed B cell lines, after treatment with mitomycin c, were pulsed with $30 \mu\text{g/ml}$ hMBP, washed and incubated (2×10^4 cells per well) with TCL no. 9 from patient DM. Results are presented as in Fig. 2.

A



B

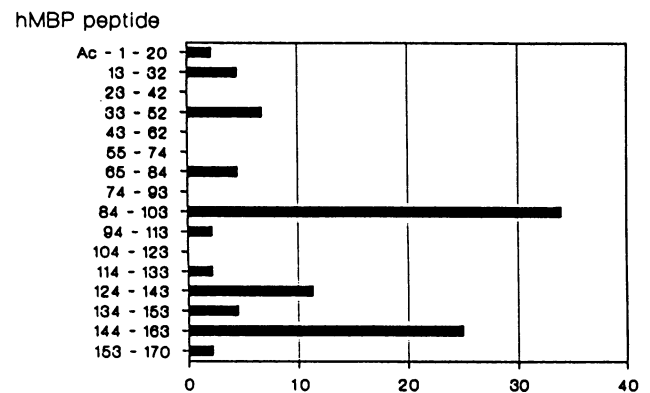


Figure 5. hMBP epitopes recognized by hMBP-specific TCL from DRB1*1501-positive or -negative MS patients. Results are expressed as percentage of TCL responding to a given hMBP peptide. (A) Peptides ($n = 34$) recognized by TCL ($n = 26$) from DRB1*1501-positive MS patients ($n = 9$). (B) Peptides ($n = 44$) recognized by TCL ($n = 35$) from DRB1*1501-negative MS patients ($n = 11$).

DQ rather than DR molecules. Furthermore, in MS patients heterozygous for DRB1*1501 hMBP-specific TCL can also be restricted by the other DR allele.

This complexity has probably multiple causes. First, most hMBP peptides bind to some HLA class II molecules, accounting for recognition, at the population level, of practically all hMBP epitopes by hMBP-specific TCL. In this respect, hMBP may be considered as a continuum of T cell epitopes, making it difficult to identify those who may be associated with the activation of pathogenic autoreactive T cells. This problem is emphasized by the observation that multiple hMBP epitopes are recognized by hMBP-specific TCL derived from either normal

donors or MS patients (26–32), presumably reflecting lack of thymic negative selection by this sequestered antigen.

Second, at least three hMBP peptides exhibit degenerate binding, accounting for the immunodominance of T cell epitopes corresponding to these sequences. This is likely to be a major reason for the blurred relationship between hMBP epitopes recognized by TCL and HLA class II molecules restricting the response. Thus, even if the hMBP peptide centered on the sequence 84–103 binds with highest affinity to DRB1*1501, it is equally well recognized by TCL from DRB1*1501-positive or -negative MS patients. This would suggest that if hMBP is the autoantigen in MS and 84–103 a

Table VI. HLA Class II Molecules Presenting hMBP Epitopes to hMBP-specific TCL

Patient	DRB1*	DQA1*	DQB1*	Line no.	hMBP peptide	Restriction
BV	1302, 07	0102, 0201	0604, 0201	11	84–103	DRB1*1302
BJ	0101, 0401	0101, 0301	0501, 0302	10	144–163	DRB1*0101
					153–170	
LA	0401, 1101	0301, 0501	0302, 0301	8	84–103	DRB1*1101
					144–163	
				21	84–103	DRB1*1101
SI	1101, 10	0101, 0501	0501, 0301	2	33–52	DRB1*1101
				4	84–103	DRB1*1101
SP	1501, 1301	0103, 0102	0602, —	9	144–163	DRB1*1301
				10	144–163	DRB1*1301
				38	33–52	DRB1*1501 (DRB5*0101)
DM	1501, 0401	0102, 0301	0501, 0302	9	84–103	DRB1*1501 (DRB5*0101)
					144–163	
				42	104–123	DRB1*1501 (DRB5*0101)
				58	84–103	DRB1*1501 (DRB5*0101)
					144–163	
SJ	1501, —	0102, —	0602, —	1	13–32	DQA1*0102/DQB1*0602
				28	104–123	DQA1*0102/DQB1*0602
TB	1501, —	0102, —	0602, —	2	13–32	DQA1*0102/DQB1*0602
				7	84–103	DRB1*1501 (DRB5*0101)
				9	84–103	DRB1*1501 (DRB5*0101)

relevant epitope, the real susceptibility gene(s), although in significant linkage disequilibrium with DRB1*1501, lie elsewhere in the HLA region. In this respect, interesting candidates for disease susceptibility genes could be represented by the polymorphic genes encoding proteins associated to peptide transport (Tap-1, Tap-2) and to the proteasome (Lmp-2, Lmp-7), since polymorphism in *Lmp* genes may result in the production of different peptides in different individuals, and polymorphism in *Tap* genes may influence peptide loading of HLA molecules (65).

Third, our evaluation of hMBP epitopes recognized by TCL from MS patients was conducted, as in any study of this type, on PBL and therefore the T cells infiltrating the inflammation site in the CNS were not directly assessed.

Fourth, MS was already clinically evident in all the patients tested at least 1 yr before establishment of hMBP-specific TCL, and it is expected that in the course of autoimmune diseases T cells not involved in disease induction may also become recruited (66). This obviously also applies to nonpathogenic T cells specific for hMBP epitopes.

In conclusion, the fact that hMBP 84–103 binds preferentially to the HLA class II molecules encoded by alleles associated to increased MS frequency could be consistent with its proposed role in the pathogenesis of MS (29, 67). Because the other major dominant peptide, 144–163, binds very weakly to DR molecules encoded by MS susceptibility alleles, we favor the hypothesis that this epitope is not involved in the pathogenesis of MS (29). The finding that peptide 84–103 also binds to several DR molecules not associated with disease may explain why many MS patients do not carry the DRB1*1501 susceptibility allele. In fact, recognition of hMBP peptides by TCL from MS patients does not appear to be directly linked to the putative HLA susceptibility alleles, because very similar hMBP immunodominant epitopes are recognized by TCL from DRB1*1501-positive or -negative MS patients. It would be interesting to know whether selective immunotherapies of established MS based on hMBP as autoantigen (2) may influence the course of disease, although, considering all this complexity, the approach may prove more problematic than originally anticipated.

Acknowledgments

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