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Research Article

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Myelin Basic Protein-specific T Lymphocyte Repertoire in Multiple Sclerosis

Complexity of the Response and Dominance of Nested Epitopes Due to Recruitment of Multiple T Cell Clones

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Abstract

The human T cell response to the myelin basic protein (MBP) has been studied with respect to T cell receptor (TCR) usage, HLA class II restriction elements, and epitope specificity using a total of 215 long-term MBP-specific T cell lines (TCL) isolated from the peripheral blood of 13 patients with multiple sclerosis (MS) and 10 healthy donors. In most donors, the anti-MBP response was exceedingly heterogeneous. Using a panel of overlapping synthetic peptides spanning the entire length of human MBP, at least 26 epitopes recognized by human TCL could be distinguished. The MBP domain most commonly recognized was sequence 80–105 (31% of MS TCL, and 24% of control TCL). Sequence 29–48 was recognized more frequently by control-derived TCL (24%) than by TCL from MS patients (5%). The MBP epitopes were recognized in the context of DRB1*0101, DRB5*0101, DRB1*1501, DRB1*0301, DRB1*0401, DRB1*1402, and DRB3*0102, as demonstrated using a panel of DR gene-transfected L cells. The TCR gene usage was also heterogeneous. Vβ5.2, a peptide of which is currently being used in a clinical trial for treatment of MS patients, was expressed by only one of our TCL. However, within this complex pattern of MBP-specific T cell responses, a minority of MS patients were found to exhibit a more restricted response with respect to their TCL epitope specificity. In these patients 75–87% of the TCL responded to a single, patient-specific cluster of immunodominant T cell epitopes located within a small (20-amino acid) domain of MBP. These nested clusters of immunodominant epitopes were noted within the amino acids 80–105, 108–131, and 131–153. The T cell response to the immunodominant epitopes was not monoclonal, but heterogeneous, with respect to fine specificity, TCR usage, and even HLA restriction. In one patient (H.K.), this restricted epitope profile remained stable for > 2 yr. The TCR β chain sequences of TCL specific for the immunodominant region of HK are consistent with an oligoclonal response against the epitopes of this region (80–105). Further, two pairs of identical sequences were established from TCL generated from this patient at different times (June 1990 and June 1991), suggesting that some TCL specific for the immunodominant region persisted in the peripheral repertoire. The possible role of persistent immunodominant epitope clusters in the pathogenesis of MS remains to be established. (*J. Clin. Invest.* 1993. 92:2633–2643.) Key words: autoimmunity • MHC class II genes • HLA-DR antigen • T cell receptors • Immunodominant epitopes

Introduction

There are several reasons to consider myelin basic protein (MBP)[1] as a candidate autoantigen involved in the immunopathogenesis of multiple sclerosis (MS) (1, 2). MBP is a strong encephalitogen in many animal species, and MBP-specific T cells have been found in experimental (3) and human postviral encephalitis (4). The frequency of MBP-reactive T cells is increased in the cerebrospinal fluid of MS patients (5), and MBP-specific T cells in the blood of MS patients display increased rates of somatic mutation, presumably reflecting sustained cell proliferation (6). Further, T cell receptor (TCR) Vβ-Dβ-Jβ motifs similar to those found in MBP-specific T cell lines (TCL) were amplified from an MS brain (7).

Although these arguments are indirect (6, 8, 9), they justify the intensive research devoted to the human T cell response against MBP over the past years. A key issue raised by these studies is the heterogeneity of the human T cell response to MBP. Unlike the response to MBP in inbred animal strains, there seems to be a broader diversity of TCR usage and epitope specificity in MS patients (10–17).

Here we present the analysis of a large panel of MBP-specific long-term TCL generated using the "split well" primary cloning technique. We found in most cases an amazing variety of MBP epitopes, restricting MHC class II molecules, and TCR elements involved in the human T cell response against MBP. However, in some MS patients the MBP-specific T cell response was directed against specific domains of the molecule that contained a cluster of immunodominant epitopes. In one patient, reactivity against the individual immunodominant epitope cluster has been observed for 2 yr, and identical TCR

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1. *Abbreviations used in this paper:* MBP, myelin basic protein; MS, multiple sclerosis; TCL, T cell line; TCR, T cell receptor.

Vβ sequences have been found in TCL generated at different time points.

Methods

Peripheral blood cells. PBMC were obtained by centrifugation of standard discontinuous density gradients (Nycomed, Oslo, Norway) from 13 patients with laboratory-supported definite MS (18), and from 10 healthy donors. Sex, age, HLA-types, and clinical details are listed in Table I.

Purification of human MBP. Adult human brain tissue was obtained within 24 h after death and frozen at −80°C. MBP was purified according to established protocols (19). Briefly, homogenized brain matter was subjected sequentially to delipidation, acid extraction, neutralization, ammonium sulfate precipitation, and acetone precipitation. Purity of the MBP preparations was assessed by gel electrophoresis.

Synthesis of MBP peptides. Peptides were synthesized using an automatic peptide synthesizer (431A; Applied Biosystems, Inc., Foster City, CA) according to the fluorenyl methoxycarbonyl method and purified by FPLC (Pharmacia, Freiburg, Germany) applying a reverse phase system. The following overlapping peptides covering the entire human MBP molecule were synthesized as confirmed by amino acid analysis: 1–20, 7–26, 16–38, 29–48, 38–58, 50–68, 61–82, 71–89, 80–99, 86–105, 94–117, 108–131, 124–141, 131–145, 139–153, 148–162, 153–170.

Isolation of MBP-specific TCL. MBP-specific TCL were established using the "split-well method," which allows the rapid isolation of clonal populations of antigen-specific $CD4^+$ TCL (20). Briefly, 2×10^5 PBMC were seeded in 200 μl medium (RPMI supplemented with 2 mM L-glutamine, 100 U/ml of penicillin, 100 μg/ml of streptomycin [all from Gibco, Berlin, Germany]), and 5% pooled human AB serum in 96-well round-bottomed microtiter plates (Nunc, Roskilde, Denmark) in the presence of 30 μg/ml MBP. Every 3–4 d the cells were fed with 100 μl medium containing 3 U recombinant IL-2 (rIL-2; Hoffmann-La Roche Inc., Basel, Switzerland). After 2–3 wk the contents of each well were split between two wells, MBP was added to one of the wells, and 2×10^5 irradiated autologous PBMC were added to both wells. The cultures were scanned microscopically for antigen-specific proliferation. Specifically responding populations were selected for further expansion.

Characterization of TCL by flow cytometry. Supernatants of hybridoma cultures (American Type Culture Collection, Rockville, MD) were used as the source for mAbs recognizing CD3 (OKT3), CD4 (OKT4), CD8 (OKT8), and HLA-DR (L243). The mAbs against TCR V region determinants came from T Cell Sciences (Cambridge, MA). These antibodies bind to Vβ5.1 (LC4), Vβ5.2 + Vβ5.3 (1C1), Vβ5.3 (W112), Vβ6.7 (OT145), Vβ8 (16G8), and Vβ12 (S511). The mAb against Vβ13.3 was purchased from Immunotech (Marseille, France). The mAb against Vβ2 (21) was kindly provided by Dr. Bernard Malissen (Marseille, France). FITC-labeled goat anti-mouse antibodies (Dianova, Hamburg, Germany) were applied as secondary reagent. The cells were evaluated by cytofluorography (Becton Dickinson & Co., Heidelberg, Germany).

Proliferation assay. Autologous PBMC (2×10^5/200 μl; 5,000 rad irradiation) were plated into 96 U-shaped wells, and TCL (2×10^4) were added. All cultures were set up as duplicates or triplicates. Human

Table I. Gender, Age, Disease Course, and HLA Type of the Blood Donors

Initials	Sex	Age	Year of diagnosis	Course	HLA-A	HLA-B	HLA-C	HLA-DQ	HLA-DR
	yr								
MS patients									
B.M.	F	26	1990	1	2/28	27/51	w3	w2/w3	4‡/w53
C.F.	F	44	1987	2	1/2	7/w62	w3/w7	w1/w2	2/w14/w52
E.S.	F	45	1971	2	2/3	18/29	w7	w1/w2	2/3/w52
E.M.	F	36	1985	2	2/28	7/35	w4/w7	w1/w2	2/7/w53
C.A.	M	25	1990	1	2/28	18/51	w3	w1/w3	1/w11/w52
B.L.	F	50	1985	2	3/24	7/27	w2	w1/w2	2/7/w53
K.S.	F	47	1964	2	1/2	7/37	w6/w7	w1	2
H.K.	M	28	1990	1	1/23	8/49	w7	w2	3/w12/w52
M.H.	F	32	1989	1	1/3	13/44	w6	w2/w3	7/w11/w52/w53
A.S.	F	29	1988	1	2/11	35/44	w4/w5	w1/w3	1/4§/w53
S.S.	F	28	1989	1	2/32	7/37	w6/w7	w1/w3	2/w8
P.S.	M	44	1984	1	2/35	44	w6/w7	w3	4‡/5/w52/w53
F.U.	M	47	1976	2	2/25	18/51	ND	w1/w3	2/w11/w52
Healthy donors									
H.W.	M	44			2/3	7/44	w7	w1	2/w14/w52
I.M.	F	29			1	8	w6/w7	ND	2/3/w52
G.W.	F	30			2/3	7/13	w6	w1/w3	2/7/w53
F.W.	M	28			1	8/37	w6	w2	3/w11/w52
M.B.	F	24			24/28	44	w2/w5	w1	1/w13/52
C.L.	M	36			1/31	44/w60	w3/w5	w1/w3	4‡/w6/w52/w53
R.H.	M	35			2/11	7/w61	w2/w7	w3	4/w9/w53
F.P.	M	25			3/24	7/18	w7	w1/w3	2/w11/w52
R.V.	M	28			3/24	7/35	w4/w7	w1/w3	2/5/w52
B.W.	F	23			1/24	44/62	w2/w3	w1/w3	4/w6/w52/w53

1, relapsing remitting; 2, (secondary) chronic progressive. ‡§Subtyping of the DR alleles: ‡DRB1*0401; §DRB1*0407. All $DR2^+$ donors had the subtype DRB1*1501, all $DR3^+$ donors DRB1*0301. ND, not determined.

MBP was used at a final concentration of 30 μg/ml, and synthetic peptides at 10 μg/ml. After 72 h, 0.22 μCi [^{3}H]thymidine (2 Ci/mmol sp act; Amersham, Braunschweig, Germany) was added for 18 h. At initial stages of this study, proliferation was measured with a liquid scintillation counter (Packard, Frankfurt, Germany); later we used a direct β counter (Matrix TM 96 Direct Beta Counter; Packard). It should be noted that the absolute counts measured by the direct counting system are only ~ 20% of the counts obtained by conventional liquid scintillation; variability and proportions of the measured values are identical with both methods.

Analysis of HLA restriction. We used L cells transfected with cDNA for the DRα chain and cDNA for the DRβ chain defining the specificities DR1 (DRB1*0101) (22), DR2a (DRB5*0101), DR2b (DRB1*1501) (23), DR3 (DRB1*0301) (unpublished; kindly provided by Dr. Karr, Iowa City, IA), DRw6 (DRB1*1402) (24), DR4Dw4 (DRB1*0401) (25), and DRw52 (DRB3*0102) (26). Mouse thymidine kinase − cells served as controls. L cells (5×10^4) pretreated with mitomycin C or irradiation (20,000 rad) were incubated in 96-well flat-bottomed microtiter wells with 2×10^4 T cells and 30 μg MBP/ml for a total of 72 h. Proliferation was measured as described above.

Analysis of TCR sequences. Total RNA was prepared from 2–3 $\times 10^6$ T cells as described (27). Oligo(dT)-primed double-stranded cDNA was synthesized from 2–3 μg of total RNA using Moloney murine leukemia virus–derived reverse transcriptase (GIBCO BRL, Gaithersburg, MD) essentially as described by the supplier. Blunt-ended cDNA was then circularized during a 5-h incubation at room temperature with T4 DNA ligase (GIBCO BRL) in a volume of 20 μl. The ligated material (1 μl) was used as a template for an inverse PCR basically as described (28). Briefly, β chain amplifications were performed in 50-μl reaction mixtures containing 1× PCR buffer, 1 U Taq-polymerase (Boehringer Mannheim, Mannheim, Germany), constant region primers for β chains (0.5 μM), and 200 μM dNTPs. The PCR primers used are as follows: Cβ forward primer (5′-GGG TCG ACG GTG TGG GAG ATC TCT GC), and Cβ inverse primer (5′-GGA ATT CTG TCT GCC ACC ATC CTC TAT GAG). Cβ primers contain EcoRI and BglII restriction sites. PCR was done for 35 cycles (denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min). PCR products were purified by phenol extraction, precipitated with ethanol, and excessively digested with the restriction endonucleases mentioned above. Fragments of expected sizes for the cDNAs were recovered from a preparative low melting point agarose gel and ligated into M13mp18 previously cut with EcoRI and BamHI. Single plaques were picked and grown up, and recombinant phage DNA was purified for DNA sequence determination. For each T cell clone three to five independent isolates were sequenced by using the Taq cycle sequencing kit and a 373A DNA sequencer (Applied Biosystems, Inc.).

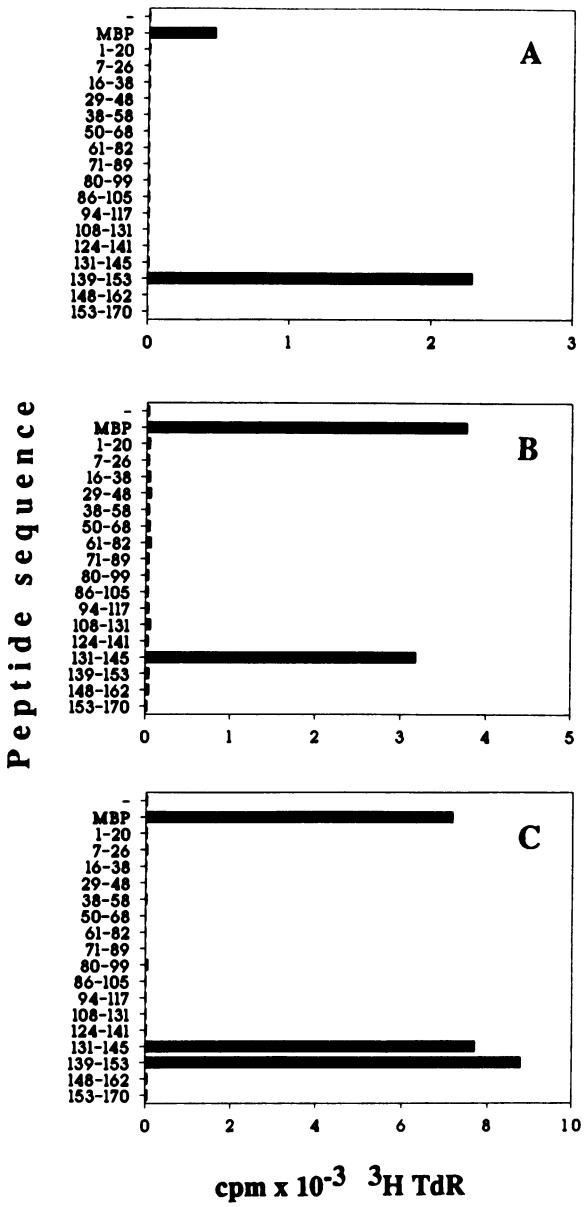

Figure 1. Peptide reactivities of three MBP-specific T cell lines from patient KS. All three lines were specific for the individual immunodominant region (131–153). T cell line KS-6 recognizes 139–153 as the only peptide (*A*), KS-12 recognizes 131–145 as the only peptide (*B*), and KS-1 recognizes both overlapping peptides (*C*). This implies that KS recognizes at least three epitopes within the region 131–153.

Results

MBP-specific TCL

In this study, we analyzed the antigen recognition of 136 MBP-specific TCL from 13 MS patients and 79 TCL from 10 healthy donors. All TCL were generated with the "split well" cloning technique using native human MBP as the selecting antigen (20). The yield of TCL using the split well technique was similar between MS patients and healthy donors. All TCL expressed the phenotype $CD3^+CD4^+HLA\text{-}DR^+$. Approximately 5% of the TCL from MS patients and from healthy donors contained a subpopulation of $CD4^+CD8^+$ double-positive cells.

Among the TCL cultured for 6–8 wk, ~ 85% were essentially monoclonal, as defined by the pattern of epitope recognition. About 85% of the individual cell lines reacted either with one single or with overlapping peptides (Figs. 1 and 2). Analysis of the TCR usage both at the mRNA level (see Table VI) (15) and at the protein level (Fig. 2) provided further evidence of homogeneity.

Complexity of the anti-MBP T cell response in MS patients and healthy subjects

EPITOPE DISTRIBUTION

In MS patients, the epitopes recognized by MBP-specific TCL were distributed over the entire length of the MBP molecule (Fig. 3 *A*). Each of the 17 overlapping peptides that we used for epitope mapping stimulated at least one TCL from at least one MS patient. The pattern of peptide reactivities suggests that a minimum of 26 different epitopes of MBP were recognized by

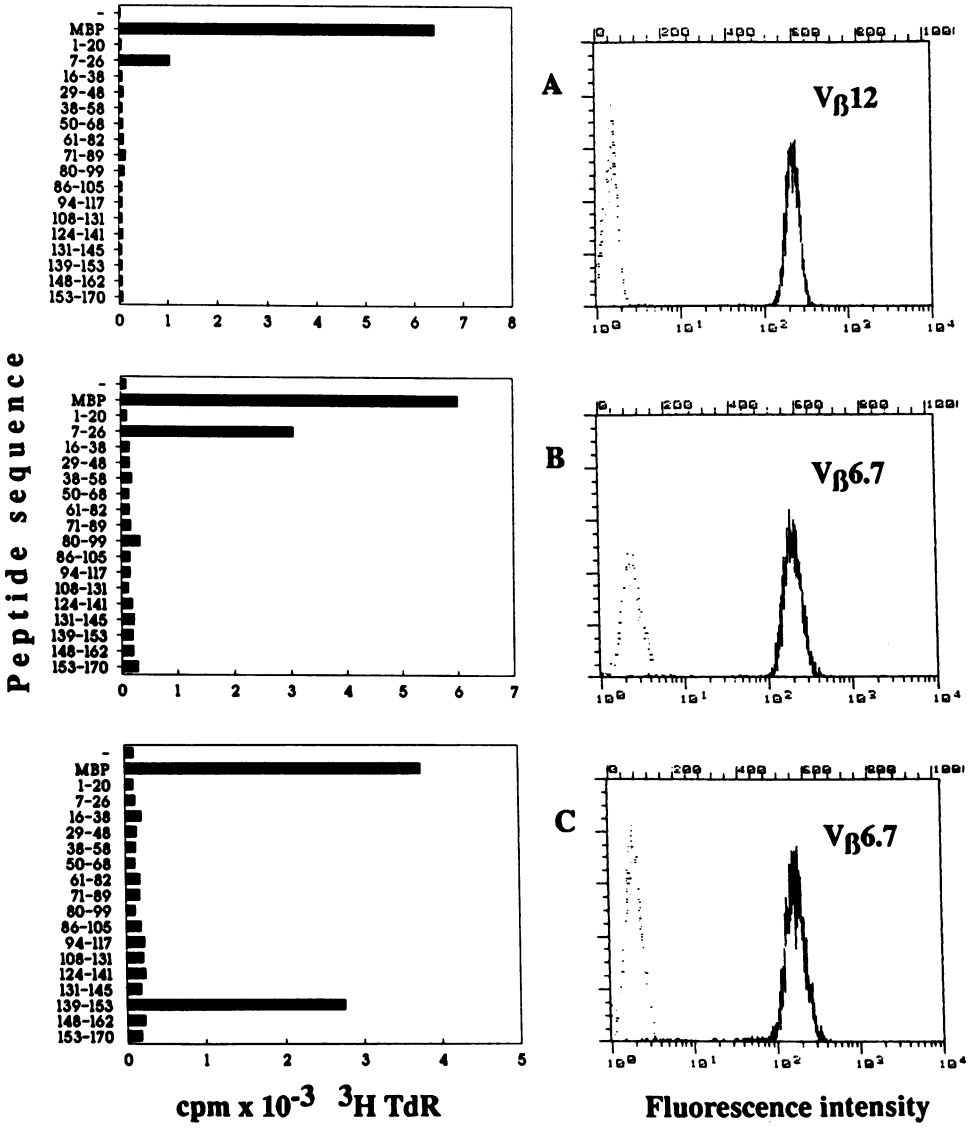

Figure 2. TCR usage by three MBP-specific T cell lines from patient BM. Peptide specificity (proliferation assay) is shown on the left, and TCR Vβ expression (FACS® analysis [Becton Dickinson & Co., Mountain View, CA]) is shown on the right. Solid lines indicate immunostaining with the indicated TCR Vβ mAb, and dotted lines represent isotype-matched negative controls. This figure illustrates two major points. First, different TCR Vβ elements may be used for recognition of the same peptide (7–26; *A* and *B*). Second, the same Vβ element (6.7) may be used for recognition of different peptides (*B* and *C*).

T cells from MS patients. 15 peptides (7–26, 16–38, 29–48, 38–58, 61–82, 71–89, 80–99, 86–105, 94–117, 108–131, 124–141, 131–145, 139–153, 148–162, 153–170) were recognized as the only peptide by at least one TCL. In addition, 11 individual combinations of overlapping peptides were recognized by at least one TCL (Table II). Furthermore, 11% of the MBP-specific TCL did not recognize any of the synthetic peptides.

Although all 17 peptides were recognized by at least one MBP-specific TCL, certain peptides were recognized more frequently than others. The most immunogenic region of MBP was located in two peptides spanning position 80–105. This region was recognized by 42 of 136 (31%) MBP-specific TCL from MS patients.

The epitopes of MBP-specific T cells from healthy donors were also broadly distributed (Fig. 3 *B*). As observed with TCL derived from MS patients, all 17 peptides were recognized by at least one TCL from at least one donor. 10 peptides (7–26, 29–48, 61–82, 80–99, 86–105, 108–131, 131–145, 139–153, 148–162, and 153–170) were recognized alone, and 7 in constant combination with an adjacent peptide (Table II). In this series, 19% of the lines did not respond to any of the peptides. This indicates that healthy donors recognize at least 18 different epitopes on MBP. The relative immunogenicity of MBP peptides was similar in MS patients and healthy donors. About 20% of all TCL isolated from healthy donors reacted against epitopes within sequence 80–105. In DR2$^+$ subjects (seven MS patients and five healthy donors), 38% of the lines from patients responded to 80–105, and 23% of the lines from controls. In striking contrast to the MS patients, a high proportion (19/79 = 24%) of MBP-specific TCL from healthy donors responded to peptide 29–48. This peptide was recognized by < 5% of the lines from MS patients (Fig. 3). This difference is significant. 7 of 10 TCL from healthy donors, but only 3 of 13 MS patients, recognized 29–48.

HETEROGENEOUS TCR USAGE

Additional evidence for the heterogeneity of the human T cell response to MBP came from the screening of 150 of the TCL from 13 MS patients with a panel of mAbs defining TCR Vβ determinants. Among these, 42 TCL (28%) were stained by one of the mAbs used; there were 11 Vβ6.7$^+$ lines, 11 Vβ12$^+$, 9 Vβ8$^+$, 6 Vβ5.1$^+$, 4 Vβ5.3$^+$, and 1 Vβ5.2$^+$. In addition we

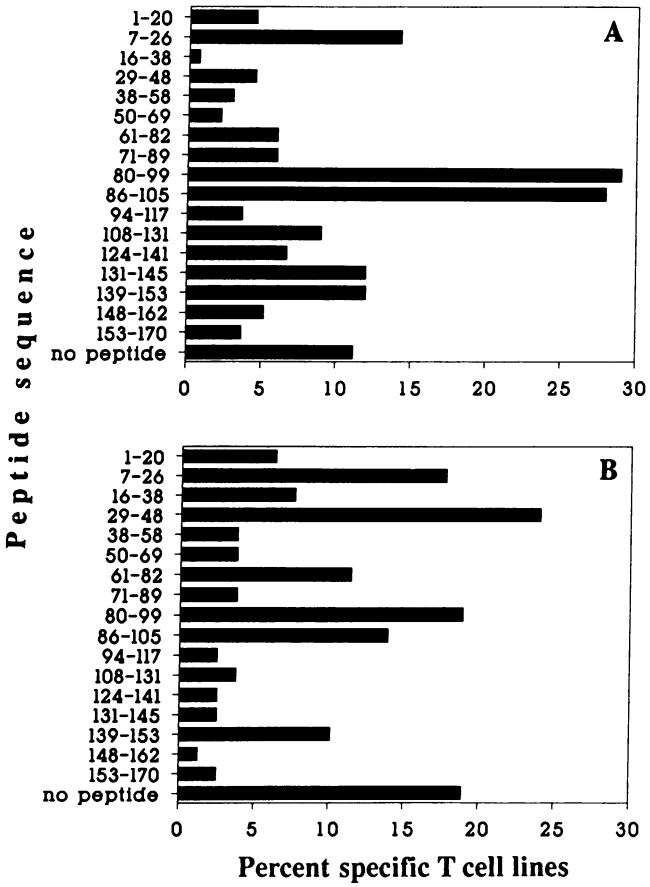

Figure 3. Overview of the peptide reactivities of MBP-specific T cell lines derived from MS patients (*A*) and healthy donors (*B*). 136 T cell lines from 13 MS patients and 79 lines from 10 healthy donors were analyzed. Each of the 17 overlapping peptides of MBP stimulated MBP-specific T cells from MS patients and healthy donors. MS patients most frequently recognized the region represented by the two peptides spanning 80–105. This region was recognized less frequently by healthy donors. The peptide 29–48 was more frequently recognized by healthy donors.

identified Vβ2- and Vβ13.3-expressing T cell lines. Further evidence of TCR heterogeneity is provided by the TCR Vβ sequences shown in Table VI. Individual patients used up to five (mAb analysis) or eight (sequence analysis) TCR Vβ elements for recognition of MBP. A similar extent of heterogeneity of TCR Vβ usage was observed in the TCL from healthy subjects.

HETEROGENEITY OF HLA RESTRICTION

In MS patients six different MHC restriction elements of MBP-specific T cells were identified, namely, DRB1*0101, DRB5*0101, DRB1*1501, DRB1*0301, DRB1*0401, and DRB3*0102. The MHC restriction elements identified in normal donors were DRB1*0101, DRB5*0101, DRB1*1501, DRB1*0301, and DRB1*1402 (Table III).

Table III further documents that one HLA restriction element (e.g., DRB5*0101) presented up to six epitopes. Conversely, one peptide (e.g., 80–99) was presented by up to four distinct HLA molecules. There was a striking difference in the number of epitopes presented by the two nonallelic products of DR2, DRB5*0101 and DRB1*1501. 12 DRB5*0101-restricted lines recognized 6 epitopes (38–58, 80–99/86–105, 108–131, 131–145, 139–153, or 131–145/139–153). In contrast, the 14 DRB1*1501-restricted lines recognized only 3 epitopes (61–82, 80–99/86–105, plus an epitope not included in our peptide panel). Note that DRB1*1501-restricted recognition of peptides 80–99/86–105 was seen both in patients and control donors.

The four DRB1*0401-restricted TCL recognized 80–99/86–105 and 108–131, and the five DRB3*0102-restricted TCL recognized an epitope represented by the overlapping

Table II. T Cell Epitopes Defined by Recognition of Overlapping Peptides in MS Patients and Healthy Donors

Peptides	No. of lines from MS patient	No. of lines from healthy donor
1–20/7–26	6	2
7–26/16–38		2
16–38/29–48		1
29–48/38–58	2	1
50–68/61–82	3	3
61–82/71–89	3	
80–99/86–105	18	9
86–105/94–117	1	
94–117/108–131	1	
124–141/131–145	4	1
131–145/139–153	4	
148–162/153–170	1	

Table III. HLA Restriction and Peptide Specificty of MBP-specific T Cells from MS Patients and Healthy Donors

HLA restriction*	Donor‡	No. of T cell lines§	Antigen specificity
DRB1*0101	HD(M.B.)	1	139–153
DRB1*0101	MS(C.A.)	1	139–153
DRB1*0101	MS(C.A.)	3	Only MBP
DRB5*0101	HD(I.M., H.W.)	2	139–153
DRB5*0101	MS(S.S.)	1	80–99/86–105
DRB5*0101	MS(B.L.)	2	38–58
DRB5*0101	MS(C.F.)	2	108–131
DRB5*0101	MS(K.S.)	2	139–153
DRB5*0101	MS(K.S.)	1	131–145
DRB5*0101	MS(K.S.)	1	131–145/139–153
DRB1*1501	HD(R.V.)	1	Only MBP
DRB1*1501	HD(I.M., G.W.)	3	80–99/86–105
DRB1*1501	MS(E.S., S.S., B.L., K.S.)	8	80–99/86–105
DRB1*1501	MS(C.F.)	2	61–82
DRB1*0301	HD(I.M.)	1	29–48
DRB1*0301	MS(E.S.)	2	29–48
DRB1*0401	MS(B.M.)	2	108–131
DRB1*0401	MS(B.M.)	2	80–99/86–105
DRB1*1402	HD(H.W.)	1	29–48
DRB3*0102	MS(H.K.)	5	80–99/86–105

The initials of the patients are shown in brackets. * MHC restriction determined with DR-transfected fibroblasts used as APC. ‡ HD, healthy donor; MS, MS patient. § Pooled data for different donors.

Table IV. Distribution of T Cell Epitopes on Human MBP in Individual Subjects

Peptide of hMBP	1–20	7–26	16–38	29–48	38–58	50–68	61–82	71–89	80–99	86–105	94–117	108–131	124–141	131–145	139–153	148–162	153–170	No peptide	No. of lines	Blood sample attained*
MS patients																				
C.A.			1						1	1					1	1		3	6	
C.F.				1		1	1					2							5	
M.H.				2				1	3	1			5	7	3			3	17	
H.K.									5	5					1				6	6/90
									6	7								1	8	6/91
									2	2									2	3/92
									1	1									1	5/92
B.L.					2				2	2						1	2		6	
B.M.	5	11					1		3	3	1				1			3	20	6/90
		1				2	3	1			2	2	2		1				7	3/92
E.M.	1	3						1	1	1		1			1	1	2		8	
A.S.		1										6				2			8	
E.S.				3	2				4	4	1								8	
K.S.								1	3	3				7	6			1	12	
P.S.		2					2	4	3	1				2	2	2	1	4	14	
S.S.							1		6	5	1	1	2						8	
F.U.		1																	1	
Healthy donors																				
M.B.	1	1	1	1				1							1			2	4	
R.H.		5	2							1					2			1	8	
C.L.		1					1												2	
I.M.				1	1				2	1					1				4	
F.P.	1	1	2	4	1				1	1			1	2		1			7	
R.V.		1		1			1											1	4	1/90
	2	2	1	4	1		2		2	1								1	10	11/91
G.W.	1	1		2		3	3	1	4	2	1		1						8	
F.W.									2	1					1			3	6	
H.W.		2		2				1	3	3		1						4	11	6/90
				4			1		1	1					1			1	7	8/90
												2			2			2	6	3/92
B.W.																	2		2	

The number of independently generated lines reactive with a particular peptide is indicated. Some lines react with several peptides, so that the number of peptides recognized is higher than the number of T lines. *Date of blood sampling (only shown for donors who were sampled on repeated occasions).

peptides 80–99/86–105 (Table III), the three DRB1*0301-restricted TCL recognized peptide 29–48 (Table III), and the one DRB1*1402-restricted TCL recognized peptide 29–48. Further, peptide 29–48 was recognized in DR3- and DRw14-negative donors (Tables I and IV), suggesting that this peptide can be presented by at least three HLA class II molecules.

Epitope distribution and clusters in individual patients and healthy donors

DIVERSE EPITOPE RESPONSE

The complexity of human T cell epitopes of MBP, which was so far described on the population level, was also noted within individual donors, MS patients, and healthy volunteers alike. For example, the MBP-specific TCL isolated from patient BM recognized 11 peptides, those from patients EM and PS responded to 9 peptides. The MBP-specific TCL isolated from individual healthy donors also recognized numerous peptides. For example, the TCL from donors GW and FP recognized 10 peptides (Table IV).

DOMINANCE OF NESTED EPITOPES

In a minority of MS patients (30% of our patients), the anti-MBP response was concentrated within a very small segment of the MBP molecule (Table V). We defined such immunodominant epitope "clusters" as sites where at least 75% of the MBP-specific TCL from one donor reacted with either one, or with two, overlapping MBP peptides. Such immunodominant cluster regions were identified in four patients. Epitope clustering was observed both in patients with newly diagnosed MS (H.K.) and in patients with long-standing disease (K.S.). Epitope clusters were seen in three distinct regions of MBP: sequences 80–105, 108–131, and 131–153. Further, the same cluster region (80–105) was observed in patients with different HLA-DR types, and, conversely, patients sharing DR2 showed immunodominant epitope clusters in different regions of MBP (Tables I and V).

PERSISTENCE OF DOMINANT EPITOPE CLUSTERS

In two patients and two healthy donors, we examined the MBP epitope profiles longitudinally. In the one case with unequivocal epitope clustering, patient H.K., five of six independent TCL isolated from a blood sample in June 1990 were specific for region 80–105 (represented by the synthetic peptides 80–99 and 86–105). In June 1991, we generated a second series of MBP-specific TCL from the same patient. This time, seven of eight TCL were specific for the same region, 80–105. A blood sample taken in March 1992 yielded only two TCL, but both recognized 80–105, and the same region was recognized by one line established in May 1992 (Tables IV and V).

Of note, the first TCL panel was established during an acute clinical exacerbation, which led to the clinical diagnosis. Since then, the patient has remained in clinical remission, although serial magnetic resonance imaging documented active lesion development over the past 7 mo. A longitudinal study of another MS patient and two normal donors showed that no immunodominant cluster appeared over time (Tables IV and V).

CLONAL HETEROGENEITY IN DOMINANT ANTI-MBP T CELL RESPONSES

T cell epitopes. The peptide response profiles recognized by TCL indicate that the immunodominant regions identified in our study are composites of several distinct epitopes. This is illustrated in Fig. 1. In patient K.S., for example, the dominant MBP sequence 131–153 can be resolved into three different epitopes recognized by three different TCL. Similarly, region 80–105 (patients H.K. and S.S.) contains two different epitopes.

Table V. Individual Immunodominant Clusters

	Most frequently recognized region	Blood sample obtained	No. of lines specific for this region/No. of lines examined (%)
MS patients			
H.K.	80–105	6/90	5/6 (83)
	80–105	6/91	7/8 (87)
S.S.	80–105		6/8 (75)
A.S.	108–131		6/8 (75)
K.S.	131–153		9/12 (75)
E.S.	80–105		5/8 (62)
M.H.	131–153		10/17 (58)
B.M.	7–26	6/90	11/20 (55)
	61–82	3/92	3/7 (42)
E.M.	7–26		3/10 (33)
B.L.	38–58; 80–105; 153–170		2/6* (33)
P.S.	71–89		4/14 (29)
C.A.	Diverse		1/6 (16)
C.F.	108–131		2/5 (40)
Healthy donors			
R.H.	7–26		5/8 (62)
F.P.	29–48		4/7 (57)
H.W.	No peptide	6/90	4/11 (36)
	29–48	8/90	4/7 (57)
	No peptide; 108–131; 139–153	3/92	2/6* (33)
G.W.	80–105		4/8 (50)
F.W.	No peptide		3/6 (50)
R.V.	Diverse	1/90	1/4 (25)
	29–48	11/91	4/10 (40)
M.B.	No peptide		2/4 (50)

At least four T cell lines were generated from each donor at each time point. * Number of T cell lines recognizing one of the indicated MBP regions.

TCR usage. Analysis of TCR Vβ expression with anti-Vβ mAbs indicated that the TCL recognizing dominant MBP epitope clusters expressed different TCR Vβ elements. Conversely, T cells using the same Vβ recognized different peptides (Fig. 2).

Table VI shows the TCR β chain sequences of 18 TCL from three MS patients (H.K., B.M., S.S.). The TCL from patients H.K. and S.S. were specific for the individual immunodominant region 80–105. The TCL from patient B.M. recognized the peptide 7–26. As indicated above, we have generated MBP-specific TCL from patient H.K. on several occasions. In this patient region 80–105 has remained immunodominant for at least 2 yr. We had the opportunity to compare TCR β chain sequences from TCL generated in June 90 (Table VI, HK1 lines) with sequences from TCL generated in June 91 (HK2 lines). In two instances, identical sequences were obtained from TCL isolated on separate occasions (Table VI, * and ‡). Further, three of the HK2 sequences were identical (Table VI, §). We observed identical TCR sequences only in the panel of

Table VI. Sequences of TCR β Chains

Tcl	Specificity	Vβ		NDN	Jβ	
HK1-5	80–105	2	CSA	RALLAGGL	YEQ	2.7*
HK1-7	80–105	5.1	ASS	LLGDG	ETQ	2.5‡
HK2-3	80–105	9.1	CAS	RPDRGL	YNE	2.1§
HK2-6	80–105	5.1	ASS	LLGDG	ETQ	2.5‡
HK2-8	80–105	9.1	CAS	RPDRGL	YNE	2.1§
HK2-9	80–105	2	CSA	RALLAGGL	YEQ	2.7*
		9.1	CAS	RPDRGL	YNE	2.1§
HK2-17	80–105	9.1	ASS	QDLWNIA	NYG	1.2
BM-5	7–26	3.1	ASS	LNRLD	GIH	1.2
		7.2	ASS	QDSGP	YNE	2.1
BM-6	7–26/1–20	2.3	CSA	ARGDN	QPQ	1.5
BM-9	7–26/1–20	3.1	ASS	PTPPGLAGVL	EQY	2.7
BM-20	7–26	6.7	CAS	ISDRAM	NTE	1.1
		w22‖	CAS	RELVG	ETQ	2.5
BM-26	7–26	1.1	ASS	PNTGLAGAA	ETQ	2.5
		12.2	FCA	ISEYGWD	QPQ	1.5
BM-27	7–26/1–20	2.3	CSA	PMDRGH	EQY	2.7
BM-29	7–26	6.7	ASS	LALGLKSY	EQY	2.7
BM-32	7–26	12.3	ASS	LGLVSY	QET	2.5
		12.2	FCA	SGPDRGH	QPQ	1.5
SS-8	80–105	13‖¶	CAS	RTGQGV	ETQ	2.5
SS-19	80–105	13‖**	ASS	GVFGGGV	DTQ	2.3
SS-22	80–105	12.4	ASS	YPPGTVPMGN	QPQ	1.5
		w22‖	ASS	EK	ETQ	2.5

TCR β chain sequences of 18 TCL from 3 MS patients (H.K., B.M., S.S). In patients H.K. and S.S., at least 75% of the TCL recognized the individual immunodominant region 80–105. In patient B.M., 55% of TCL recognized region 7–26 (cf. Table V). For each patient, all TCL analyzed here were specific for the same peptide. In patient B.M., some but not all TCL specific for peptide 7–26 crossreacted with peptide 1–20, indicating that the TCL recognized different epitopes in the immunodominant region. In patient H.K., TCL were generated at different times (HK1: TCL generated in June 1990; HK2: TCL generated in June 1991). Shown are the Vβ and Jβ segments (nomenclature according to reference 53) expressed by the TCL and the amino acid sequences of the N-D-N region (one letter code). Several TCR Vβ transcripts were amplified from some of the TCL, indicating that these TCL were oligoclonal rather than monoclonal. *‡§ Note that in patient H.K., some TCR sequences were identical. Sequence identity was established on the nucleotide level (not shown). ‖ Nomenclature according to (54). ¶ Vβ from clone IGRB16 (54). ** Vβ from clone IGRB14 (54).

TCL from patient HK, not in the TCL from patients B.M. and S.S., which were analyzed in the same PCR experiments. In line with the analysis of TCR Vβ expression using anti-Vβ mAbs, none of the β chain sequences was Vβ5.2.

HLA restriction. The diversity of the TCL recognizing dominant epitope clusters was confirmed by their heterogeneous DR restriction patterns. For example, in patient H.K., five TCL specific for the immunodominant region 80–105 were all restricted by DRB3*0102; the restriction of the remaining TCL from H.K. could not be identified with our panel of L cell transfectants (Table III). In patient S.S., among the six TCL specific for the immunodominant region 80–105, three TCL were restricted by DRB1*1501, and one line was restricted by DRB5*0101 (Table III and Fig. 4).

Discussion

In this study we have used a technique that allows the isolation of multiple (oligo-)clonal T cell lines directly from the PBMC (20). This method is exceptionally well suited to analyze the human autoimmune T cell repertoire. It allows many TCL to be established from a single blood sample, and moreover, many of these TCL are monoclonal. Thus, this approach permits a more direct insight into the actual T cell repertoire to be obtained, by avoiding the in vitro selection/competition events that are innate to bulk cultures and secondary cloning technique (J. Lannes-Vieira and H. Wekerle, manuscript in preparation).

We found that in most MS patients, and in all normal donors, the anti-MBP response used a large variety of T cell clones recognizing a broad spectrum of peptide epitopes, and expressing a diversity of TCR genes. However, in a few cases of MS the polyclonal T cell response was focused to circumscript segments of the MBP molecule. In one patient (H.K.), the immunodominance of one region of MBP (80–105) has persisted for at least 2 yr. Several TCL established from this patient in June 1990 and June 1991 expressed identical TCR β chain sequences.

Our study confirms and extends previous work from our own group (20, 29), and from other laboratories (30–33), that revealed that the spectrum of MBP epitopes recognized by T lymphocytes is much more diverse in humans than in inbred rodents (34). Our present data document that T cell epitopes can be located in virtually all regions of the human MBP se-

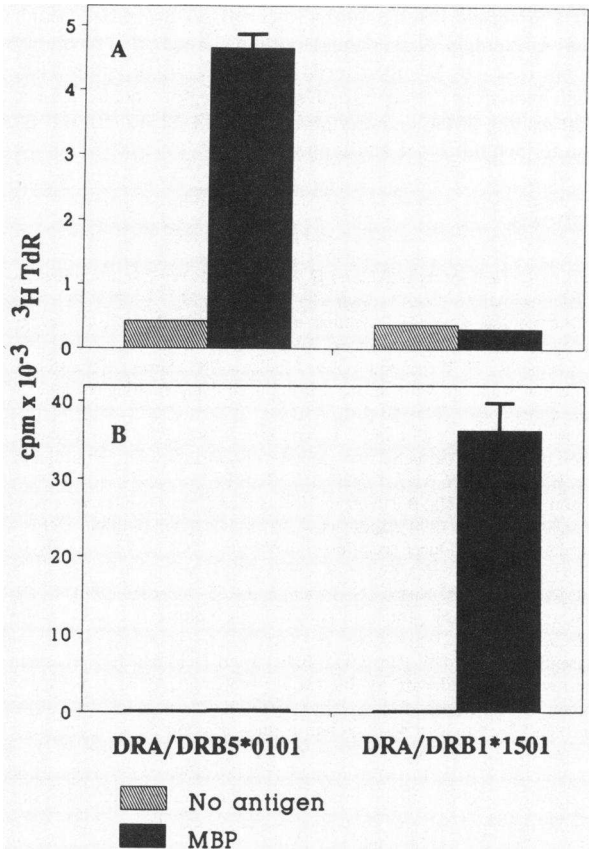

Figure 4. HLA restriction analysis of two T cell lines from patient SS. Both lines were specific for the individual immunodominant region (80–99/86–105). The lines used different HLA molecules as restriction elements. T cell line SS-8 is restricted by DR2a (DRA/DRB5*0101) (*A*), and T cell line SS-9 is restricted by DR2b (DRA/DRB1*1501) (*B*). Restriction was established with DR2a(DRA/DRB5*0101)- and DR2b(DRA/DRB1*1501)-transfected L cells.

quence. We identified a minimum number of 26 epitopes, a number that still presumably grossly underestimates the actual epitope diversity.

In agreement with the published data (30, 31), we found that peptides spanning MBP positions 80–105 were recognized by a notably high number of TCL. There was no obvious association of this peptide with the HLA-DR2 haplotype, in line with another report (35). Furthermore, there was no significant difference in the frequency that TCL recognizing this region were selected from MS patients or healthy donors. On the other hand, our present study identified a peptide (MBP 29–48) that was more frequently recognized by T cells from normal donors than by T cells from patients. PBMC from 7 of 10 normal donors (17/79 TCL), but only 3 of 13 patients (6/134 TCL) were specific for this peptide, which could be presented by at least three HLA molecules. We note that this observation is consistent with the data presented in a previous study (31), in which reactivity to a similar peptide (amino acids 31–50) was found more frequently in healthy donors than in MS patients.

For the analysis of HLA restriction we used HLA-DR-transfected mouse L cells. This technique has the advantage that in positive cases it allows the straightforward and unequivocal identification of an HLA restriction element. One disadvantage is that even L cells expressing appropriate DR restriction molecules may fail to stimulate TCL because they are lacking human adhesion molecules (36, 37). Our analysis of HLA restriction demonstrates that the number of HLA class II products presenting MBP epitopes was large. Further, we found three new restriction elements for MBP-specific T cells, including the supertypic determinant DRw52 (DRB3*0102). Yet, epitope diversity was by no means merely due to the diversity of HLA class II products used for antigen presentation. In fact, one DR heterodimer (DRA/DRB5*0101) presented at least six distinct MBP epitopes, more than described previously (29, 37). Finally, we noted marked peptide "promiscuity" with individual peptides being presented by multiple HLA class II products (38). Peptide 86–105, for example, was presented by DRB5*0101, DRB1*1501, DRB1*0401, and DRB3*0102 in our analysis, and DRB1*0408 and DRw13Dw19 in the work by Martin et al. (13). This astounding promiscuity of many MBP peptides for class II products has been recently documented by direct binding studies (39).

The TCR V gene repertoire used by MBP-specific human TCL is a controversial issue. Some investigators have emphasized that the usage of TCR V genes is skewed, but there was no consensus about the actual Vβ gene dominating the human response (10, 16, 17). Others have pointed to a more diverse TCR usage (14, 15, 35). In our panel of MBP-specific TCL we observed a high degree of heterogeneity of TCR Vβ usage. Each Vβ element that could be identified with the available panel of mAbs was used by MBP-specific T cells from MS patients. No dominance of any particular Vβ element shared by any discernible group of patients was evident. Note, however, that the panel of anti-TCR Vβ mAbs that is presently available allows the identification of < 50% of the Vβ families expressed by human T cells. Thus, a restricted or skewed TCR usage could have escaped this type of analysis.

In our group of patients only 1 of 150 MBP-specific TCL, which were analyzed with anti-TCRVβ mAbs, expressed Vβ5.2. This is of particular relevance in view of attempts to use peptides from TCR variable chains (e.g., Vβ5.2) for the treatment of MS patients (40).

In a minority of MS patients, at least 75% of TCL responded either to one peptide, or to peptides tightly nested within very narrow segments of the MBP molecule. The dominant epitopes differed between individual donors and were preferentially located in MBP sequences 80–105, 108–131, and 131–153. Also, in normal donors, different MBP-specific TCL may recognize the same peptide. However, the most frequently recognized peptides varied between TCL generated at different times from the same donor (see H.W. and R.V. in Table 5).

In one MS patient (H.K.) studied longitudinally, T cell reactivity against one dominant MBP sequence has been observed for > 2 yr. In an initial screening we found that five of six TCL isolated from PBMC recognized MBP sequence 80–105. When reexamined after an interval of 1 yr, seven of eight TCL responded against the same peptide, and this dominance could be traced further after another 9 and 12 mo. Similar evidence of persistence of immunodominant epitopes in individual patients has recently been seen in another study (41).

Interestingly, in this patient we found identical TCR β chain sequences in TCL generated at different times (June 1990 and June 1991; see Table VI). Further, three of the seven sequences from TCL raised in June 1991 were identical. The fact that we found these sequences only in TCL from patient H.K. but not in TCL from B.M. and S.S., which were analyzed

in the same PCR experiments, argues against the possibility that the sequence identities represent PCR artifacts. We favor the interpretation that these sequences derive from (80–105)-specific TCL that were present in the blood of H.K. for an extended period of time and were probably clonally expanded. This would be consistent with previous evidence for clonal expansion of MBP-specific TCL in the blood of MS patients (6).

Although the first set of TCL from patient H.K. was isolated during a clinical attack, and although magnetic resonance imaging documented persisting disease activity, a direct connection between the clinical course and the TCL response pattern remains yet to be shown.

A recent report (7) has described sequence motifs shared between TCR Vβ5.2-Dβ-Jβ gene rearrangements in brain lesions of MS patients and MBP-specific TCL obtained from blood. We did not observe any of these motifs in our panel of TCR sequences from MBP-specific TCL. However, this may not be surprising, as none of our sequenced TCL expressed Vβ5.2 (Table VI).

The nature and implications of the persistent MBP epitope dominance that we observed in some MS patients are not at present understood. It is clear, however, that the T cell response to immunodominant epitopes is by no means monoclonal. In our study, TCL recognizing dominant peptides were heterogeneous with respect to peptide fine specificity, TCR usage, and MHC restriction. This is in line with previous reports (14, 15, 35) demonstrating heterogeneous TCR β gene rearrangements of T cells responding against identical MBP peptides.

Recognition of dominant epitopes is commonly observed in rodent experimental autoimmune encephalitis, where it seems to be a genetically controlled feature of immune reactivity. In H-2^u mice, for example, almost all encephalitogenic T cells recognize MBP epitopes within sequence 1–11, whereas in H-2^s mice epitopes nested in sequence 89–109 appear to dominate the MBP-specific T cell response (34).

The epitope dominance in Lewis rats (42, 43) and PL/J mice (44) has two features, which may be relevant to understand the epitope dominance observed in our MS patients. First, we found recently that in the Lewis rat, epitope dominance is not a property of the naive T cell repertoire, but develops upon immunization in vivo. TCL isolated from MBP-primed rats show specificity to epitopes in sequence 68–88, whereas TCL from naive animals display much broader epitope patterns (J. Lannes-Vieira and H. Wekerle, manuscript in preparation). Second, in rodents with MBP-induced experimental autoimmune encephalitis, the initial monospecific MBP response seems to degenerate with time and, in addition, T cell responses to other myelin autoantigens develop (45, 46).

It is tempting to speculate that the examples of persistent epitope dominance that we observed in the T cell repertoire of some MS patients might be involved in the pathogenesis of the disease. The strictly dominant epitope recognition, with no "epitope spreading" (47) over time, could reflect a sustained, active immune process. Clearly, this possibility has to be corroborated by additional evidence before immunotherapies can be targeted against the TCL recognizing clustered epitopes (48–52).

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