

Increased Frequency of Interleukin 2-responsive T Cells Specific for Myelin Basic Protein and Proteolipid Protein in Peripheral Blood and Cerebrospinal Fluid of Patients with Multiple Sclerosis

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Summary

Equal numbers of CD4⁺ T cells recognizing myelin basic protein (MBP) and proteolipid protein (PLP) are found in the circulation of normal individuals and multiple sclerosis (MS) patients. We hypothesized that if myelin-reactive T cells are critical for the pathogenesis of MS, they would exist in a different state of activation as compared with myelin-reactive T cells cloned from the blood of normal individuals. This was investigated in a total of 62 subjects with definitive MS. While there were no differences in the frequencies of MBP- and PLP-reactive T cells after primary antigen stimulation, the frequency of MBP or PLP but not tetanus toxoid-reactive T cells generated after primary recombinant interleukin (rIL-2) stimulation was significantly higher in MS patients as compared with control individuals. Primary rIL-2-stimulated MBP-reactive T cell lines were CD4⁺ and recognized MBP epitopes 84–102 and 143–168 similar to MBP-reactive T cell lines generated with primary MBP stimulation. In the cerebrospinal fluid (CSF) of MS patients, MBP-reactive T cells generated with primary rIL-2 stimulation accounted for 7% of the IL-2-responsive cells, greater than 10-fold higher than paired blood samples, and these T cells also selectively recognized MBP peptides 84–102 and 143–168. In striking contrast, MBP-reactive T cells were not detected in CSF obtained from patients with other neurologic diseases. These results provide definitive *in vitro* evidence of an absolute difference in the activation state of myelin-reactive T cells in the central nervous system of patients with MS and provide evidence of a pathogenic role of autoreactive T cells in the disease.

Multiple sclerosis (MS)¹ is a chronic inflammatory disease of the central nervous system (CNS) (1). Although the etiology and the pathogenesis of the disease are still unknown, there is increasing evidence suggesting that MS is a T cell-mediated autoimmune disease of the CNS white matter (reviewed in reference 2). T cell responses to myelin basic protein (MBP) and proteolipid protein (PLP), the two most abundant myelin proteins, are thought to have pathological importance in MS (reviewed in reference 3). This postulate is supported both by the prominent myelin-specific inflam-

mation induced by activated but not resting T cells specific for MBP and PLP in experimental autoimmune encephalomyelitis (EAE) and by their pathological role in postinfectious encephalomyelitis in humans (4, 5).

Recent experiments have indicated that many organ-specific autoreactive T cells are not deleted in the thymus and circulate at a low frequency in the periphery (6). These autoreactive T cells may exist without pathologic consequences as they can be isolated from individuals without autoimmune disease. Common to T cells reactive to other autoantigens, autoreactive T cells specific for MBP are present in both patients with MS and normal individuals (7). They occur at a relatively low frequency in peripheral blood mononuclear cells, not differing significantly between MS patients and normal individuals (7, 8). The average precursor frequency of MBP-specific T cells is 10⁻⁶, similar to that of T cells against exogenous antigens typically found in blood of nonim-

¹ Abbreviations used in this paper: CNS, central nervous system; CSF, cerebrospinal fluid; EAE, experimental autoimmune encephalomyelitis; LDA, limiting dilution analysis; MBP, myelin basic protein; MS, multiple sclerosis; OND, other neurologic diseases; PLP, proteolipid protein; TT, tetanus toxoid.

munized individuals, which increases by >40-fold after immunization (9).

Recent studies in human have demonstrated that T cell responses to MBP are preferentially directed at two immunodominant regions, residues 84–102 and 143–168 of human MBP (7, 8, 10, 11). It has also been demonstrated in the EAE model that T cell responses to encephalitogenic epitopes can be found in unprimed healthy animals without provoking the disease (12). The frequency of MBP-specific T cells in the peripheral blood of rats with adoptively transferred relapsing EAE averages only 1.6×10^{-5} during the first relapse and 0.5×10^{-5} during the second relapse (13). Thus, antigen recognition of autoreactive T cells to the encephalitogenic epitopes by itself is not sufficient to induce the disease. Rather, encephalitogenic T cells must acquire other functional characteristics in order to cross the blood-brain barrier and to mediate autoimmune process in the CNS. The major characteristic of encephalitogenic T cells that mediate EAE is their activated state since resting T cells are inaccessible to the brain compartment. Thus, we postulate that the myelin-reactive T cells in the blood of patients with MS will be in a different state of activation as compared with that of controls.

A limiting dilution analysis (LDA) was performed comparing primary stimulation of T cells with rIL-2 to either MBP, PLP, or tetanus toxoid (TT) to determine the frequency of activated T cells recognizing myelin antigens in peripheral blood and CSF of patients with MS, subjects with other neurologic diseases (OND), and normal controls. Additionally, MBP-reactive wells were selectively expanded to short-term T cell lines and reactivity to overlapping MBP epitopes examined. An increased frequency of MBP and PLP but not TT-reactive T cells generated by primary rIL-2 stimulation was observed in blood of MS patients as compared with controls. MBP-specific T cell lines were exclusively CD4⁺ and preferentially recognized epitopes within residues 84–102 and 143–168 of MBP. Most importantly, MBP-specific T cells accounted for ~7% IL-2-responsive T cells derived from cerebrospinal fluid (CSF) of MS patients, which was >10-fold higher than paired blood samples. In contrast, MBP-specific T cells were not detectable in CSF samples of patients with OND. These results provide definitive *in vitro* evidence of an absolute difference in the activation state of myelin-reactive T cells in the CNS of patients with MS and provides compelling evidence to suggest a pathogenic role of autoreactive T cells in MS.

Materials and Methods

Reagents. Human MBP was purified from the white matter of the human brain by the method previously described by Deibler et al. (14). The same MBP preparation was used in all experiments described in this study. The MBP peptides were synthesized by the Merrifield solid phase method and were purified by high pressure liquid chromatography. TT was obtained in purified form from the Massachusetts Public Health Laboratory (Boston, MA). rIL-2 was obtained from EuroCetus Inc. (Amsterdam-Zuidoost, The Netherlands). T cell stimulatory activity of this rIL-2 was titrated as compared with recombinant preparations of human IL-2 obtained from

other sources (Boehringer Mannheim Biochemicals, Indianapolis, IN; and Genzyme Corp., Cambridge, MA). A concentration of 50 U/ml of rIL-2 (EuroCetus, Inc.) was comparable to 5 U/ml of Boehringer Mannheim Biochemicals rIL-2 in activation of resting T cells. Lectin-free IL-2 purified by delectinated affinity chromatography from PHA-stimulated T cell culture supernatant was purchased from Collaborative Biomedical Products (Bedford, MA). Autologous heat-inactivated serum was used for the initial 7 d of stimulation, followed by culture with human serum obtained from Sigma Chemical Co. (St. Louis, MO). Media consisted of RPMI (Gibco Laboratories, Grand Island, NY) supplemented with 10% sera and was otherwise as previously described (7). TT was obtained from Lederle-Prexiss Biologics (Pearl River, NY).

Patients and CSF Samples. A group of 62 patients with definite MS (15) characterized clinically predominantly by a relapsing-remitting course were included in this study. Patients had an average expanded disability status scale (EDSS) (16, 17) of 3.6 and had not received steroids for at least 10 mo before the study. None of the patients ever received other immunosuppression. Approximately 15–25 ml of CSF was removed by lumbar puncture from 20 patients with MS and 7 patients with other neurologic diseases for diagnostic purposes. Cells used in the experiments were collected by centrifugation of CSF at 1,200 rpm for 10 min within 30 min after lumbar puncture. Informed consent was obtained from each patient, and the protocol was approved by the Human Subject Committee at Brigham and Women's Hospital. Lumbar punctures were obtained on patients with other neurologic diseases, and these are listed in Table 1.

LDA of Myelin Antigen-specific T Cell Lines from Peripheral Blood by Primary Stimulation with Myelin Antigens or rIL-2. PBMC were prepared from heparinized venous blood by Ficoll gradient separation. PBMC of each subject were used for two sets of LDA with either primary rIL-2 or primary MBP, PLP, or TT stimulation. For primary myelin-antigen stimulation, PBMC were seeded at limiting dilution at 2×10^5 , 10^5 , and 5×10^4 cells per well (60 wells for each cell density) in U-bottomed plates (Costar Corp., Cambridge, MA) and stimulated with either human MBP (40 µg/ml) or PLP (50 µg/ml). To stimulate TT-specific T cells, PBMC were plated by limiting dilution at 10^5 , 5×10^4 , and 2.5×10^4 cells per well in the presence of 12.5 limit of flocculation/ml of TT. For primary IL-2 stimulation, the same cell preparations were plated at 2×10^4 , 10^4 , and 5×10^3 cells per well (60 wells for each cell concentration) in the presence of 50 U/ml of rIL-2. It should be noted that these plating cell concentrations were predetermined to give optimal clonal response to the antigens. Antigen-stimulated wells were cultured without the addition of IL-2.

On day 8 each well was scored for antigen reactivity by the following procedure. 10^5 irradiated (5,000 rad) autologous PBMC pulsed with either MBP, PLP, or TT (final concentration, 2.5 Lf/ml) in 200 µl of media without rIL-2 were added to each well after removing the culture supernatant. PBMC were pulsed by incubating with 100 µg/ml of MBP, PLP, or 12.5 Lf/ml of TT, respectively, at 37°C for 4 h and washing away free antigens before irradiation. 50 U/ml of rIL-2 was added to each well on day 10, and antigen reactivity was measured in each well by a split well assay on days 12–14 (4–6 d after the second stimulation with antigen-pulsed PBMC) to determine which wells contained T cells responding specifically to MBP, PLP, or TT. An aliquot of each culture well was split into four aliquots (~ 10^4 cells per aliquot) and two wells were cultured in the presence of 10^5 autologous PBMC pulsed with the respective antigen and two control wells received unpulsed PBMC. Cells were cultured for 72 h and then pulsed with 1 µCi of [³H]thymidine per well during the last 16 h of culture and sub-

sequently harvested by an automated cell harvester (Betaplate 1295-004; Pharmacia Fine Chemicals, Piscataway, NJ). [³H]thymidine uptake was measured in a beta scintillation counter (betaplate 1205; Pharmacia Fine Chemicals).

Wells were scored positive if the mean Δ cpm (cpm T cell line with APC pulsed with antigen - CPM T cell line APC alone) was >1,000 cpm and exceeded the control CPM by at least three times. The number of antigen-reactive wells did not exceed 16% of the total wells tested, which implies by the Poisson probability that an antigen-specific T cell line derived from a well is most likely to originate from a single precursor cell and represents a monoclonal response to the antigen. This was confirmed by subsequent cloning experiments indicating that specific T cell clones derived from a T cell line were all identical as to their reactivity to a single antigenic peptide and their identical pattern of TCR V gene usage.

Estimation of the frequency of antigen-specific T cells was performed by applying the Poisson Formula (18, 19), in which $Fr = (u^r/r!) \times e^{-u}$, where Fr is the probability of obtaining r -specific T cells in a well when the average number of precursor cells per well is u at a given concentration. The fraction of negative wells per total number of wells is given by $F_0 = e^{-u}$; when $u = 1$, $F_0 = 0.37$. Therefore, theoretically, when the average number of responding T cells per well is one, 37% of the wells will be scored as negative. Extrapolation to this point in limiting dilution gives a number of cells, the reciprocal of which represents the frequency of the antigen-specific cells in question.

LDA of MBP-specific T Cells and IL-2-responsive T Cells in CSF and Paired Blood Samples. To perform a LDA to determine the frequencies of IL-2-responsive T cells and MBP-specific T cells in CSF compared with paired blood samples, a different protocol was designed to activate IL-2 responsive T cells by two rounds of IL-2 stimulation. The protocol allowed effective stimulation of a limited number of T cells obtainable from each lumbar puncture. Specifically, CSF cells and paired PBMC were counted in duplicate in a hemocytometer and plated at three different cell concentrations, depending on the number of cells obtained from each CSF sample. Typically, cells were seeded at 400, 200, and 100 cells per well by limiting dilution and were cultured in the presence of 10^5 irradiated autologous PBMC and 10% lectin-free IL-2 (~40 U/ml). This concentration of lectin-free IL-2 was predetermined to give optimal cloning efficiency of T cells at given concentrations. After 7 d, fresh IL-2 with autologous PBMC were added to each well. On day 14, all wells were scored microscopically as to whether they were growth positive and tested for reactivity to MBP. The same split-well protocol as described above was used to determine which wells contained MBP-reactive T cells using MBP-pulsed and control PBMC. The frequencies of IL-2 responsive and MBP-specific T cells were analyzed by the Poisson statistics as described above.

Generation of MBP-specific T Cell Lines from Blood/CSF and Peptide Analysis. To examine the peptide reactivity of each MBP-reactive well, aliquots of MBP-reactive wells were restimulated with irradiated PBMC pulsed with MBP in the presence of IL-2, as previously reported (7). 10^4 cells of each MBP-specific T cell line were cultured with 10^5 irradiated autologous PBMC or EBV-transformed B cells pulsed with the respective peptides. Peptide-pulsed APC were prepared by incubating PBMC or B cells with 40 μ g/ml of peptide or a peptide mix for 4 h and washed two times before irradiation. Cells were cultured for 72 h and then pulsed with 1 μ Ci of [³H]thymidine per well during the last 16 h of culture and harvested as described above.

Flow Cytometry. Phenotypic expression of the T cell lines was analyzed by means of direct immunofluorescence dual staining. 10^5 T cells were prewashed with ice-cold PBS containing 2% fetal calf

serum and incubated with monoclonal antibodies directed at CD4, CD8, CD45RA (2H4), and CD45RO (UCHL-1) conjugated with fluorescein or phycoerythrin (Coulter Immunology, Hialeah, FL; and Dakopatts, Copenhagen, Denmark) on ice for 30 min. Cells were then washed twice before flow cytometric analysis. Background fluorescence reactivity was determined with phycoerythrin or fluorescein-conjugated mouse Ig. Flow cytometric analysis was performed by using an Epics C flow cytometer (Coulter Electronics, Hialeah, FL) with gates set to read total cell populations.

Results

Frequency Analysis of Resting and Activated MBP- and PLP-specific T Cells. The clinical characteristics and HLA-DR and HLA-DQ haplotypes of MS patients and control subjects are shown in Table 1. The frequency of MBP-specific T cells was determined by LDA at three cell concentrations predetermined to give maximal and clonal T cell response to MBP (<16% positive wells). PBMC were initially stimulated under different activation conditions with either MBP or IL-2 in 19 patients with MS and 17 normal subjects. Our pilot experiments revealed that all cell lines grown in rIL-2 expressed CD3 phenotype and TCR- α/β , reflecting the T cell lineage. As shown in Fig. 1, the precursor frequency of T cells specific for MBP was 1.1×10^{-6} in patients with MS and at 0.9×10^{-6} in normal subjects. As previously observed, this difference was not statistically significant (Fig. 1, A vs. C; $p > 0.5$). In contrast, the frequency of MBP-reactive T cells generated by primary culture with rIL-2 was higher than the precursor frequency of MBP-reactive T cells in 14 of 19 patients with MS, as compared to 4 of 17 normal subjects. The mean frequency of IL-2-generated T cells specific for MBP was 3.2×10^{-6} in patients with MS and was 1.2×10^{-6} in normal donors (Fig. 1, B vs. D; $p < 0.02$).

The frequency of IL-2-generated T cells specific for PLP was also examined in a series of patients. As with MBP, the precursor frequency of activated PLP-reactive T cells generated by primary rIL-2 stimulation was higher than that generated after primary PLP stimulation in patients with MS (1.3×10^{-6}) as opposed to control subjects (0.3×10^{-6} ; Fig. 1, F-H; $p < 0.02$). To examine whether the increased frequency of rIL-2-autoreactive T cells was specific for myelin antigens, the precursor frequencies of TT-reactive T cells were similarly determined. As shown in Fig. 1, J and L, and in contrast to MBP and PLP, the frequency of TT-specific T cells generated by primary rIL-2 stimulation represented only 12–18% of TT-reactive T cells in both MS patients ($n = 7$) and normal subjects ($n = 5$) who had been immunized with TT >5 yr before the assay. These results indicate that the increased frequency of rIL-2-responsive autoreactive T cells is specific for MBP and PLP myelin antigens.

Phenotypic Analysis of MBP-reactive T Cells. 93 MBP-reactive T cell lines generated by either MBP or IL-2 stimulation were examined for cell surface expression of CD4, CD8, CD45RA, and CD45RO determinants. With one exception, all of the MBP-reactive T cell lines were CD4⁺CD8⁻. These CD4⁺CD8⁻ T cell lines could be subgrouped into two subsets according to their CD45RA expression. 36 of 93 MBP-

Table 1. HLA Type and Clinical Characteristics of Patients with MS and OND and Normal Subjects

Subject	Age/Sex	EDSS	Diagnosis	Sampling	HLA-DR	HLA-DQ
MS-1	40/F	2.0	RR	PB	DR2,DR7	DQW1,DQW3
MS-2	43/M	6.0	RR	PB	DR2 (DRB1-1501)	N.T.
MS-3	42/F	2.5	RR	PB	DR2,DR4	N.T.
MS-4	37/F	2.0	RR	PB	DR2,DRW11,DRW52	DQW1,DQW7
MS-5	38/F	6.0	CP	PB	DR2,DR7,DRW53	DQW1,DQW2
MS-6	42/F	6.0	RR	PB	DR2 (DRB1-1501)	N.T.
MS-7	31/F	1.5	RR	PB	DR2 (DRB1-1501)	N.T.
MS-8	30/F	0	RR	PB	DR2	N.T.
MS-9	42/F	3.5	RR	PB	DR2 (DRB1-1501)	N.T.
MS-10	31/F	0	RR	PB	DR2,DR7,DRW53	DQW1,DQW2,
MS-11	38/F	4.0	RR	PB	DR1,DR2	N.T.
MS-12	31/F	2.0	RR	PB	DR2,DR7,DRW53	DQW1,DQW2
MS-13	41/F	6.0	RR	PB	DR2 (DRB1-1501)	N.T.
MS-14	45/F	7.5	CP	PB	N.T.	N.T.
MS-15	40/M	1.5	CP	PB	DR2 (DRB1-1501)	N.T.
MS-16	47/M	5.5	RR	PB	DR4,DRW8,DRW52,DRW53	DQW4,DQW8
MS-17	46/M	7.5	RR	PB	DR2	DQW1
MS-18	48/F	2.5	RR	PB	DR7,DRW13,DRW52,DRW53	DQW1,DQW2
MS-19	28/F	2.0	RR	PB	DR1,DR2	DQW1
MS-20	34/F	6.5	CP	PB,CSF	DR2,DR3,DR52	DQW1,DQW2
MS-21	42/F	6.0	RR	PB,CSF	DR2 (DRB1-1501)	N.T.
MS-22	20/F	3.5	RR	PB,CSF	DR2,DR6	N.T.
MS-23	47/F	6.0	RR	PB,CSF	DR2-	N.T.
MS-24	42/F	3.5	RR	PB,CSF	DR2-	N.T.
MS-25	43/M	6.0	RR	PB,CSF	DR2 (DRB1-1501)	N.T.
MS-26	27/M	2.5	RR	PB,CSF	DR2-	N.T.
MS-27	37/F	2.0	RR	PB,CSF	DR2,DR11	N.T.
MS-28	29/F	6.5	RR	PB,CSF	DR1,DR3,DRW52	DQW1,DQW2
MS-29	31/F	0	RR	PB,CSF	DR2,DR7,DRW53	DQW1,DQW2
MS-30	46/F	6.0	RR	PB,CSF	DR2,DR3,DRW52	DQW1,DQW2
MS-31	29/F	2.0	RR	PB,CSF	DR2-	N.T.
MS-32	38/F	4.0	RR	PB,CSF	DR1,DR2	DQW1
MS-33	41/F	6.0	RR	PB,CSF	DR2 (DRB1-1501)	N.T.
MS-34	39/M	2.0	RR	PB,CSF	DR6,DR7,DRW52,DRW53	DQW1,DQW2
MS-35	31/F	2.0	RR	PB,CSF	DR2,DR7,DRW53	DQW1,DQW2
MS-36	39/F	2.5	RR	PB,CSF	DR7,DRW6,DRW52,DRW53	DQW2,DQW6
MS-37	45/F	0	RR	PB,CSF	DR1,DR2	DQW1
MS-38	38/F	1.5	RR	PB,CSF	DR2 (DRB1-1501)	N.T.
MS-39	29/F	2.5	RR	PB,CSF	DR2,DR4	

continued

specific T cell lines examined expressed both CD45RA and CD45RO isoforms (Fig. 2 A) while the other 57 T cell lines expressed only the CD45RO isoform (Fig. 2 B). As previously shown, MBP-specific T cell lines and clones expressed either CD45RO or both CD45RA and CD45RO isoforms

of the leukocyte common antigens (20, 21). The CD45 isoform expression of MBP-specific T cell lines examined in this study did not differ between patients with MS and control subjects and may be related to the T cell growth cycle at time of analysis. Of 93 short-term MBP-specific T cell lines, the

Table 1. (continued)

Subject	Age/Sex	EDSS	Diagnosis	Sampling	HLA-DR	HLA-DQ
OND-1	38/F		Neurosarcoidosis	PB,CSF	DR4,DR53,DR7	DQW2,DQW3
OND-2	27/F		Polyneuropathy	PB,CSF	DR7	DQW1
OND-3	31/F		Cerebral palsy	PB,CSF	DR1,DR7	DQW1,DQW2
OND-4	41/F		Neurosis	PB,CSF	DR2,DR6	DQW1
OND-5	29/F		Viral meningitis	PB,CSF	DR2,DRW11	DQW1
OND-6	43/M		Peripheral neuropathy	PB,CSF	DR4,DRW53	DQW3
OND-7	28/M		Vertigo	PB,CSF	DR3	DQW1,DQW3
NS-1	25/M			PB	DR2-	N.T.
NS-2	21/M			PB	DR6,DR10,DRW52	DQ1,DQ3
NS-3	32/M			PB	DR1,DR2	DQW1
NS-4	26/M			PB	DR2-	N.T.
NS-5	30/F			PB	DR7,DRW53	DQW2,DQW3
NS-6	30/F			PB	DR7,DRW53	DQ1,DQ2
NS-7	23/M			PB	DR6,DRW52	DQW1
NS-8	34/M			PB	DR2	N.T.
NS-9	25/F			PB	DR2 (DRB1-1501)	N.T.
NS-10	33/M			PB	DR11	
NS-11	26/F			PB	DR2,DR3,DRW52	DQW1,DQW2
NS-12	19/F			PB	DR3,DR6,DRW52	DQW1,DQW2
NS-13	25/F			PB	DR3	
NS-14	31/M			PB	DR2-	N.T.
NS-15	47/M			PB	DR2-	N.T.
NS-16	57/F			PB	DR4,DR9,DRW53	DQW8,3,DQW9,3
NS-17	50/F			PB	DR9,DRW13,6,DRW52,DRW53	DQW7,3,DQW9,3

N.T., not tested; RR, relapsing-remitting MS; CP, chronic progressive MS; PB, peripheral blood. DR2- is tissue-typed negative for DR2. DR2 (DRB1-1501) is defined by molecular tissue typing and refers to DR2,Dw2.

line JA1B2 was CD8⁺ (98%), CD4⁻ (<1%), and CD45RA⁻ (Fig. 2, C), and responded specifically to peptide 143–168 (Fig. 3). Attempts to clone this line to further characterize its MHC restriction were unsuccessful.

Peptide Specificity of MBP-reactive T Cells from Peripheral Blood. Reactivity to peptides derived from a sequence of the human MBP molecule were determined and data of 29 MBP-specific T cell lines, generated after primary stimulation with MBP from peripheral blood, are shown in Fig. 3, *bottom*. As previously reported, T cell lines preferentially recognized the 84–102 and the 143–168 regions of MBP.

It was of interest to compare peptide specificity of T cell lines derived by primary rIL-2 stimulation to lines derived by antigen stimulation. Specifically, peptide reactivity was examined in 132 MBP-specific T cell lines derived with MBP stimulation and 60 T cell lines grown in IL-2 (Fig. 4). T cell lines generated from each patient or normal donor reacted predominately to either peptide 84–102 or 143–168 and in some subjects both peptides. Moreover, the predominant reactivity pattern of T cell lines generated after primary rIL-2 stimulation correlated with that of MBP-derived T cell lines.

Frequency and Peptide Specificity of MBP-reactive T Cells Generated in IL-2 from CSF of Patients with MS and Patients with OND The frequency of T cell lines after primary IL-2 stimulation and their reactivity to MBP were examined in the CSF of 20 MS patients and 7 patients with OND. The clinical data and HLA-DR and HLA-DQ haplotypes of these patients are given in Table 1. Fig. 5 illustrates an example of the frequency analysis plotted according to the Poisson probability distribution for a paired blood/CSF sample. Flow cytometric analyses of >3,000 cell lines grown in rIL-2 confirmed that >98% of the lines were CD3⁺ and expressed TCR- α/β .

An increased frequency of growth-positive wells was observed after primary IL-2 stimulation that represented exclusively CD3⁺ T cells in the CSF of MS patients as compared with paired blood specimen (15.7×10^{-4} vs. 7.8×10^{-4} ; $p < 0.0001$), while there were no differences in the total numbers of inflammatory mononuclear cells in the CSF (Table 2). LDA of CSF T cells demonstrated a higher frequency of MBP-reactive T cells after primary IL-2 stimulation from patients with MS as compared with T cells from paired PBMC cultures using the same cell concentrations and procedures to generate

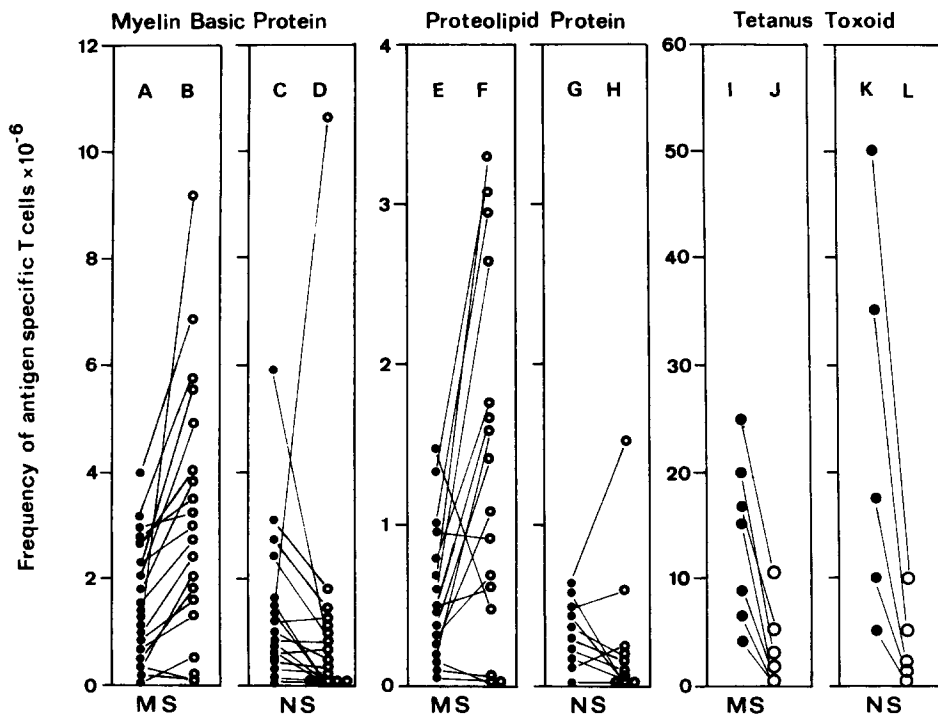


Figure 1. Frequency analysis of T cells reactive to MBP and PLP in peripheral blood of MS patients and normal subjects. 19 patients with MS and 17 normal subjects were analyzed by LDA for the frequency of T cells reactive to MBP. Similarly, 16 different patients with MS and 10 normal subjects were examined for PLP reactivity. The frequency of T cells reactive to TT was tested in seven patients with MS and five normal donors. The frequencies of antigen-specific T cells were calculated according to the Poisson statistics. Paired frequencies analyzed in each individual subject by antigen stimulation (filled circles) and IL-2 stimulation (open circles) are connected by a line. The differences of A-B, B-D, E-F, and F-H are statistically significant ($p < 0.02$).

the clones (5.4×10^{-5} vs. 0.32×10^{-5} ; $p < 0.001$). The paired values between CSF and blood are shown in graphic form in Fig. 6. These T cell lines responded specifically to MBP and expressed exclusively the CD4 phenotype. Reactivity of these clones to MBP peptides were investigated. As with peripheral blood T cells, MBP 84-102 and 143-168 were preferentially recognized by the IL-2-derived CSF T cells (Fig. 3).

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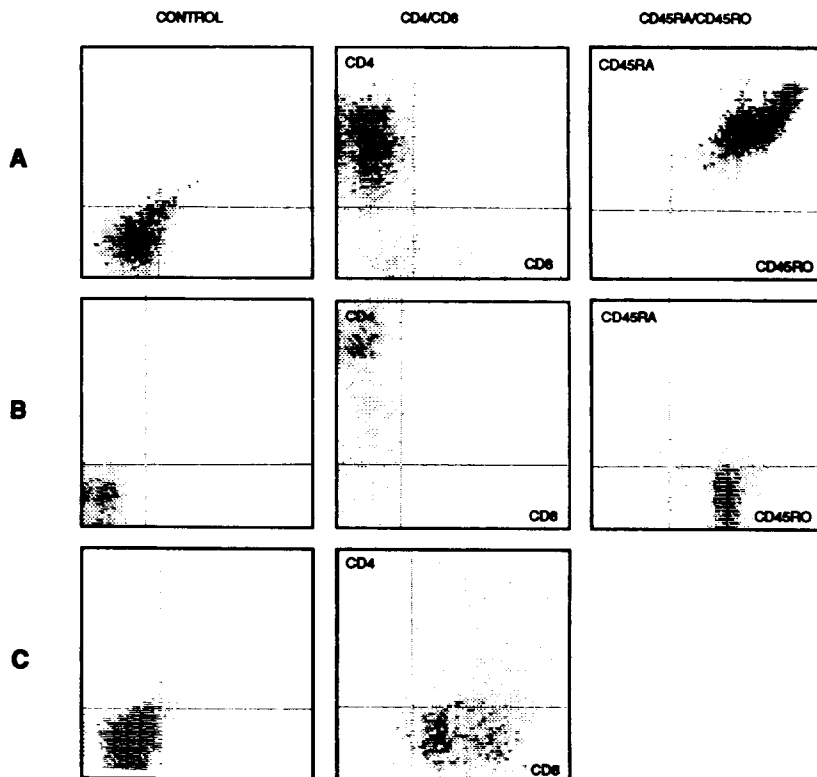


Figure 2. Flow cytometric analysis of MBP-specific T cell lines derived from patients with MS and controls. 10^5 cells of each MBP-specific T cell line were tested in a direct immunostaining for the CD4, CD8, CD45RA, and CD45RO expression. (A) 36 lines that stained positively for CD4, CD45RA (2H4), and CD45RO (UCHL-1); and (B) 57 lines that expressed CD4⁺, CD45RA⁻/CD45RO⁺ phenotypes. (C) Phenotypic profile of a CD8⁺ MBP-specific T cell line (JA1B2).

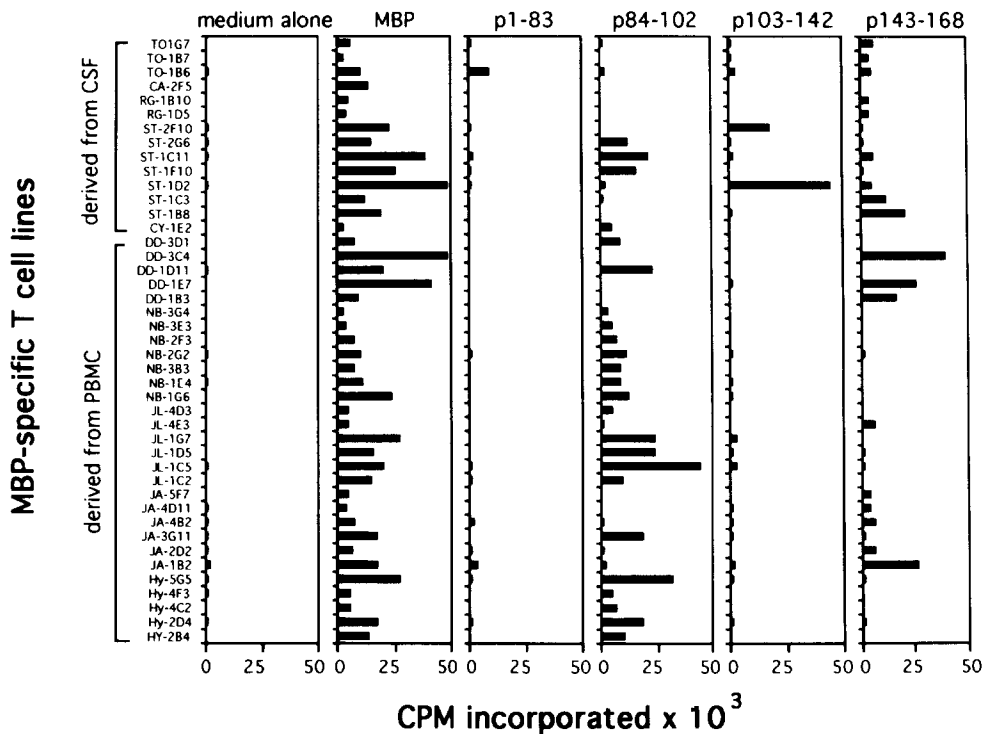


Figure 3. Peptide specificity of MBP-reactive T cell clones from blood vs. CSF. 29 MBP-specific T cell lines derived from peripheral blood by MBP or IL-2 stimulation and 14 T cell lines derived from CSF were tested for reactivity to MBP and MBP peptides. Reactivity to residues 1-83 and 103-143 of MBP was assayed by combining the peptides covering these two regions (7). The standard deviation was <15% of the mean CPM.

The frequency of growth-positive wells and MBP-reactive T cells after IL-2 stimulation from the CSF of subjects with other neurologic diseases was compared with that of patients with MS. In parallel experiments, the frequency of growth-positive wells in the CSF was found to be lower in patients with OND as compared with the patients with MS ($p < 0.001$), while there were no differences in the frequency of IL-2-responsive T cells in the peripheral blood (Table 2 and

Fig. 6), consistent with observations that the immune system is activated in patients with MS. Perhaps of greatest importance, 7% of the T cells in the CSF of patients with MS were MBP reactive and these were not detectable in CSF of any of these patients with other neurologic diseases.

Expansion of In Vivo Activated TT-reactive T Cells with rIL-2 after Immunization. It was of importance to determine whether the precursor frequency of TT-reactive cells with primary

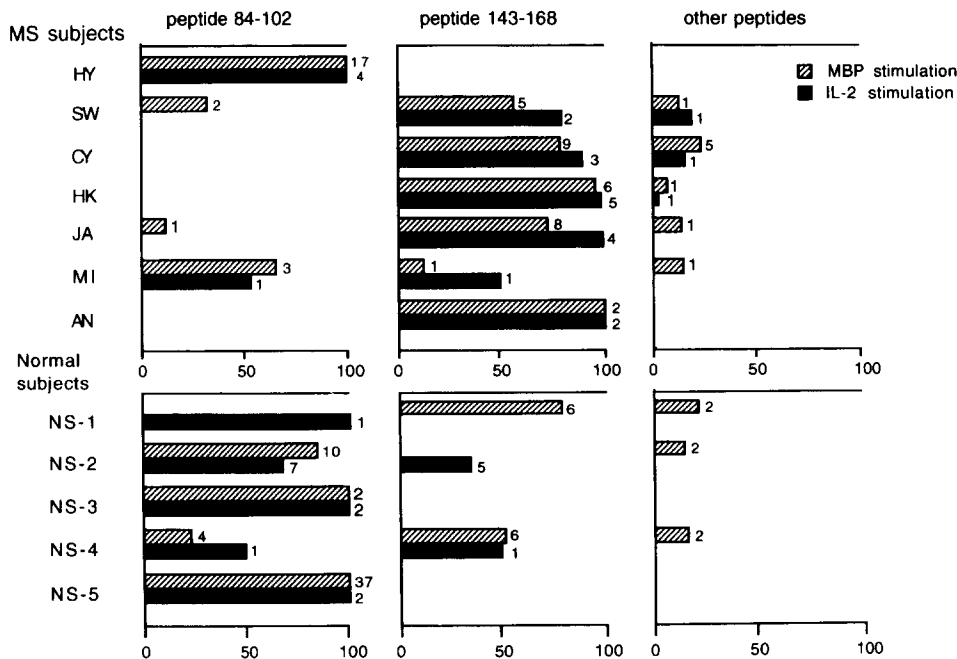


Figure 4. Comparison of peptide reactivity of MBP-specific T cell lines derived by MBP with that of T cell lines derived by primary IL-2 stimulation. Two panels of MBP-specific T cell lines (192 independent lines) subgrouped according to the original stimulus (MBP and IL-2) were tested for their peptide reactivity. Data are given as percentage of T cell lines reactive to a peptide or a peptide mix. Actual numbers of peptide-specific T cell lines are indicated on the bar.

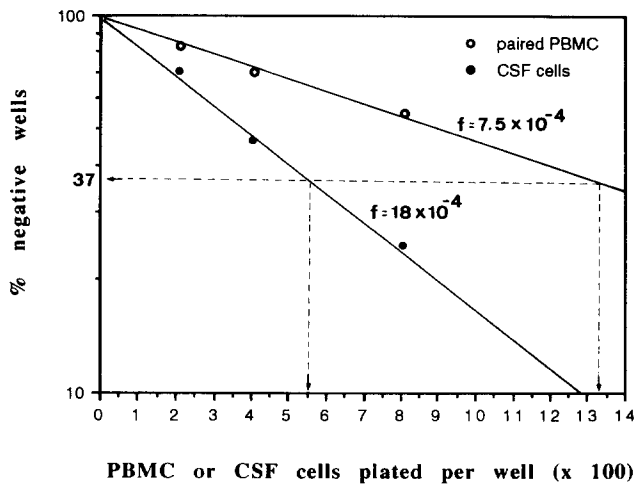


Figure 5. Representative frequency analysis of growth-positive wells with primary IL-2 stimulation in paired CSF and peripheral blood. CSF cells and PBMC were counted and plated at limiting dilution with 10^5 irradiated autologous PBMC and 10% lectin-free IL-2 preparation. The frequency (f) of growth-positive wells assessed by an inverted microscope was obtained at each cell concentration. Estimation of the frequency of IL-2-responsive T cells was analyzed according to the Poisson statistics.

rIL-2 stimulation increased after antigen immunization relative to the precursor frequency with primary antigen stimulation. Thus, we compared the frequency by LDA of antigen-reactive T cells after antigen inoculation. Five normal subjects were immunized subcutaneously with TT and the frequency of antigen-reactive T cells was calculated 5–10 d after primary stimulation with either antigen or rIL-2 using the same technique used to investigate the frequency of myelin-reactive T cells (Table 3). In two subjects, we also measured the frequency of antigen-reactive T cells prior to TT immunization.

The frequency of TT-reactive T cells with primary rIL-2 stimulation increased 1 wk after immunization. However, even as early as 5 d after immunization, the frequency of TT-reactive T cells generated with primary antigen stimulation was greater than that obtained with primary rIL-2 cloning. This is in contrast to myelin-reactive T cells in MS patients where the frequency of rIL-2-generated clones was higher than that obtained with antigen cloning.

Discussion

CD4⁺ T cells recognizing sequestered autoantigens such as MBP and PLP have been demonstrated in the circulation of normal individuals as well as in the blood of patients with the autoimmune CNS disease MS. A critical question has been whether these autoreactive T cells are pathologic in patients with MS but not in normal individuals. In the EAE model, the minimal requirements for autoimmune inflammatory disease of the CNS white matter are the presence of either MBP- or PLP-reactive CD4⁺ T cells that are in an activated as opposed to resting state. This led to the hypothesis that if myelin-reactive T cells are critical for the pathogenesis of MS, they would exist in a different state of activation as compared with myelin-reactive T cells cloned from the blood of normal individuals. We now demonstrate that the frequency of MBP and PLP but not TT-reactive T cells generated after primary rIL-2 stimulation is significantly higher in the blood and CSF of MS patients as compared with control subjects.

High affinity IL-2 growth factor receptors are transiently expressed after T cell stimulation and are associated with T cell activation. As antigen-stimulated T cells bearing high affinity IL-2R can proliferate in response to IL-2, it has been postulated that IL-2R⁺-bearing T cells represent lymphocyte populations that have undergone activation and can be expanded with IL-2 (22, 23). This was investigated after im-

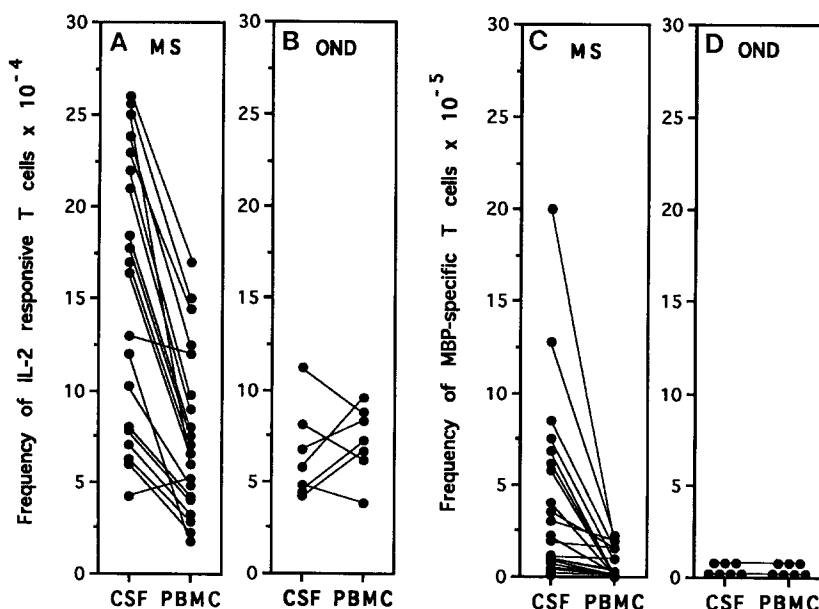


Figure 6. Frequency analysis of growth-positive wells and MBP reactivity of T cells in paired CSF and peripheral blood samples after primary IL-2 stimulation. CSF cells and paired PBMC were plated at limiting dilution in the presence of 10% lectin-free IL-2 preparation. On day 14, cultures were scored microscopically for growth positivity (*left*) and tested for proliferative response to MBP (*right*) with autologous MBP-pulsed APC in a thymidine-uptake assay.

Table 2. Frequencies of IL-2-responsive T Cells and MBP-specific T Cells in CSF and Paired PBMC of Patients with MS and OND

Subject	Cell counts/ ml CSF	Frequency of IL-2-responsive T cells		Frequency of MBP-specific T cells		
		CSF	PBMC	CSF	PBMC	
		$\times 10^{-4}$		$\times 10^{-5}$		
		(A)	(B)	(C)	(D)	
MS-20	12,240	18.0 (13.1–25.7)*	7.4 (4.1–11.8)	2.6 (1.8–6.6)	0	
MS-21	1,520	10.4 (6.1–14.7)	14.7 (10.2–20.4)	5.5 (4.3–9.2)	0	
MS-22	1,210	7.7 (4.3–12.8)	4.7 (2.9–8.8)	3.8 (2.2–7.2)	0	
MS-23	1,220	16.6 (11.5–23.7)	13.3 (9.9–19.7)	9.1 (6.9–14.8)	0	
MS-24	1,490	17.5 (13.1–24.2)	11.2 (7.8–17.2)	8.3 (5.3–14.5)	0	
MS-25	2,040	23.3 (16.9–32.4)	8.7 (6.4–13.2)	20.0 (14.8–28.0)	0	
MS-26	3,440	25.0 (18.7–33.1)	7.7 (4.2–12.4)	8.4 (6.0–13.5)	1.8 (1.4–3.2)	
MS-27	2,820	7.7 (4.3–11.9)	2.3 (1.7–5.8)	6.7 (4.2–11.8)	1.9 (1.3–4.4)	
MS-28	5,040	12.1 (8.6–17.8)	12.1 (8.8–17.4)	1.7 (1.2–4.3)	0	
MS-29	4,790	24.3 (17.2–32.2)	8.4 (6.4–13.5)	3.4 (2.3–7.4)	0	
MS-30	5,790	23.8 (16.8–32.5)	3.2 (2.2–7.8)	4.9 (2.9–8.6)	0	
MS-31	4,630	6.1 (3.6–11.1)	2.7 (1.8–5.6)	0	0	
MS-32	2,190	17.8 (13.2–24.3)	10.0 (7.8–15.4)	0	0	
MS-33	3,380	25.0 (18.7–33.5)	16.6 (11.5–23.5)	2.4 (1.7–6.9)	0	
MS-34	3,820	18.2 (13.4–24.8)	8.3 (5.8–11.4)	6.6 (5.2–10.8)	0	
MS-35	4,480	6.2 (4.3–11.8)	4.2 (2.4–7.5)	6.4 (5.2–10.7)	0	
MS-36	2,620	12.5 (8.6–18.3)	6.2 (4.6–10.8)	1.4 (1.3–4.5)	0	
MS-37	1,240	7.7 (4.2–13.1)	4.3 (2.7–9.2)	0	0	
MS-38	4,570	11.1 (7.6–17.0)	2.0 (1.4–5.6)	16.5 (11.5–24.1)	2.7 (1.8–5.2)	
MS-39	3,840	23.8 (16.9–32.0)	8.3 (6.0–12.9)	0	0	
Mean	3,620	15.7	7.8	5.4	0.32	
		(E)	(F)	(G)	(H)	
OND-1	5,220	10.4 (7.3–16.4)	9.6 (7.3–14.6)	0	0	
OND-2	2,240	7.2 (5.1–10.8)	8.3 (5.8–12.4)	0	0	
OND-3	2,410	4.6 (2.8–9.0)	7.8 (4.2–14.5)	0	0	
OND-4	5,320	4.1 (2.3–8.5)	8.7 (6.4–13.4)	0	0	
OND-5	4,380	4.8 (2.9–9.2)	6.8 (5.2–10.2)	0	0	
OND-6	1,190	3.3 (2.0–7.9)	4.2 (2.4–8.9)	0	0	
OND-7	2,230	2.1 (1.3–5.0)	4.9 (3.8–9.1)	0	0	
Mean	3,260	5.2	6.9	0	0	
Column pair	(A)–(B)	(C)–(D)	(E)–(F)	(A)–(E)	(B)–(F)	(C)–(G)
p^\ddagger	0.0001	0.0001	0.165	0.001	0.578	0.0001

* 95% confidence limits.

‡ Calculated by *t* test.

munization with TT followed by a LDA with either primary antigen or rIL-2 stimulation. The frequency of TT-reactive T cells was higher after immunization with primary rIL-2 or antigen stimulation. The frequency of TT-reactive

T cells remained higher after primary antigen as compared with rIL-2 stimulation as early as 5 d after immunization. This is not surprising as rIL-2 should stimulate only activated T cells that express high affinity IL-2Rs while antigen

Table 3. Frequency of α -TT-reactive T Cells with Immunization

Normal subjects	Primary stimulation	Pre immunization	Post immunization
		$\times 10^{-5}$	
1	TT	0	1.1
	rIL-2	0	1.1
2	TT	0	6.2
	rIL-2	0	3.8
3	TT	ND	21.1
	rIL-2		16.3
4	TT	ND	2.3
	rIL-2		1.3
5	TT	ND	3.4
	rIL-2		1.0

LDA was performed in five subjects 5–10 d after standard immunization with TT. Subjects 1 and 2 had not been immunized with TT for >15 yr.

should result in the stimulation of both resting and activated T cells.

In normal individuals, the frequency of myelin-reactive T cells was either higher or the same with primary antigen stimulation as compared with rIL-2 stimulation. In distinct contrast to the myelin response in normal individuals and the TT response in all subjects, MBP- and PLP-reactive T cells from MS patients after primary rIL-2 stimulation were found at a higher frequency compared with primary MBP and PLP stimulation. This difference in frequency of MBP- and PLP-reactive T cells after primary rIL-2 stimulation in MS patients as compared with controls indicates a distinct functional difference in the myelin-reactive T cells in diseased subjects.

What might explain the higher frequency of MBP-reactive T cells in MS patients with rIL-2 stimulation as opposed to primary antigen stimulation? One possibility is that in vivo activated T cells, which are more sensitive to apoptosis after TCR signaling and are present at a higher frequency in the blood of MS patients, would undergo cytolysis with antigen but not IL-2. This hypothesis appears to be unlikely as the frequency of TT-reactive T cells was higher with primary antigen as compared with IL-2 stimulation in subjects recently immunized with TT. An alternative hypothesis is that MBP-reactive T cells are present at a higher frequency in MS patients, but these cells are in a different state of activation in the blood and CSF of MS patients such that they proliferate to an IL-2R signal but not to a TCR signal. While this may in part be the definition of anergic T cells, whether or not these myelin-reactive T cells identified after primary rIL-2 stimulation are indeed anergic will require further investigations.

We have previously described a type of anergy for autoreactive MBP-T cells in vitro by stimulation with MBP peptides in the absence of other antigen-presenting cells, and this anergy is due to T cell antigen presentation (24, 25). T cell anergy may be induced in vivo, where MBP-specific T cells undergo clonal expansion during the disease process and become anergic when stimulated with breakdown products of MBP, which can be reversed by the addition of rIL-2 (24). Thus, clonally expanded, previously activated T cells from MS patients might be more sensitive to the induction of anergy, and the calculated frequency of MBP-specific T cells measured by MBP stimulation may not reflect the actual frequency and may be considerably underestimated. However, whatever mechanisms underlie the differences in frequencies between MBP- and PLP-specific T cells stimulated with rIL-2 or myelin antigens, these results demonstrate clear functional differences in activation requirements between myelin-reactive T cells in the blood of patients with MS as compared with normal or OND control subjects. These results are also consistent with a recent study that suggested in vivo activation of MBP-specific T cells in MS using an hypoxanthine guanine phosphoribosyltransferase (hprt)⁻ mutant assay (26).

Activated MBP-specific T cell lines derived with primary rIL-2 stimulation preferentially recognized both immunodominant MBP regions between residues 84–102 and 143–168 as MBP-stimulated T cells, and similarly expressed the same CD4⁺CD8⁻ phenotype. This raises the question as to whether a subset of the total precursor pool of autoreactive T cells become activated or whether there is an alternative pool of autoreactive T cells that undergo a separate differentiation process. To further address this question, experiments are underway to define the TCR V-D-J DNA sequences of these MBP-specific T cell clones grown in IL-2 as compared with their MBP-derived counterparts.

As MS is a disease limited to the CNS, we examined the frequency of growth-positive wells and MBP-specific T cells after primary IL-2 stimulation in the CSF of MS patients as compared with patients with OND. The frequency of growth-positive wells after primary IL-2 stimulation was higher in the CSF of patients with MS as compared with subjects with OND, while there were no differences in this frequency of IL-2-stimulated T cells in the peripheral blood. As higher concentrations of IL-2 can stimulate NK cells and lymphokine-activated killer (LAK) cells, we determined that the wells contained almost exclusively CD3⁺ T cells. Though in the CSF of MS patients ~7% of the T cells recognize MBP, the antigen reactivity of the majority of cells is unknown. Moreover, it is possible that many CD3⁺ T cells are NK cells. Nevertheless, these observations in total are consistent with the presence of an activated CNS immune system in patients with MS. Perhaps of greatest importance, a surprisingly high proportion of T cells in the CSF of patients with MS were MBP reactive and these were not detectable in CSF of any of the patients with other neurologic diseases. This may provide the most compelling evidence to date of a pathogenic role of autoreactive T cells in MS.

The difficulty in detecting MBP-reactive T cells in MS CSF was previously explained by a low frequency of MBP-reactive

T cells in the CSF (5, 27, 28). In retrospect, this observation may have been due to cloning with primary antigen stimulation, as 16 of 20 MS patients exhibited an increased frequency of MBP-reactive T cells in CSF as compared with paired blood samples with primary IL-2 stimulation. This observation is consistent with other recent studies using different methodologies that have also suggested that there is an increased number of in vivo activated MBP-specific T cells in the CSF of patients with MS (27, 28).

An important question remains as to how MBP-specific T cells are activated and accumulate in the CNS. It is possible that the increased frequency of T cells responsive to MBP in the CSF is a primary event in which myelin-reactive T cells are activated in the periphery and acquire the capability to pass through the blood-brain barrier. Once they have trafficked into the CNS, these myelin-reactive T cells may be reactivated when they recognize myelin peptides in the context of class II MHC and then recruit other inflammatory cells such as γ/δ T cells and macrophages into the CNS that may be important in mounting a substantial immune response (29). The activation of MBP-specific T cells in the periphery may be triggered without MBP, and may involve other pathways of T cell activation, such as activation by superantigens, molecular mimicry, or T-T cell activation (CD2-LFA-3/1-CAM-1-LFA-1) (30, 31). Alternatively, the primary inflammation in the CNS may not be mediated by MBP-specific T cells, and expansion of MBP-specific T cells may be secondary to the release of myelin proteins resulting from the destruction of myelin tissue.

It has been unknown whether MBP-specific T cells present in the peripheral blood and compartmentalized within the CNS originate from the same precursor population(s). That is, are MBP-reactive T cells, which are selected and expanded in the CNS, different from MBP-reactive T cells selected in the periphery? While suppression in SJL/J mice demonstrated selective expansion of MBP-reactive T cells recognizing an encephalitogenic epitope (MBP 87–99) in the spinal cord not recognized by MBP-specific T cells derived from peripheral blood (32), in MS patients, identical oligoclonal T cells can frequently be found between blood and CSF compartments (33, 34). Here, we demonstrate that independent MBP-reactive T cell clones derived from the CSF of MS patients showed a restricted reactivity pattern to the two immunodominant regions of MBP (MBP 84–102 and MBP 143–168) comparable to MBP-specific T cells derived from peripheral blood paired to the CSF. This observation is consistent with recent investigations showing sequence homologies between the TCR β chain CDR3 region of MBP-reactive T cells and V β 5.2 CDR 3 sequences isolated by PCR from the brain of patients with MS (35).

Activated encephalitogenic T cell clones selectively recognize immunodominant MBP residues. These clones are pathogenic in mice and rats and induce EAE (36, 37). We postulate that activated human T cell clones reactive to PLP and immunodominant MBP epitopes are analogous to the encephalitogenic T cells isolated from animals with EAE and thus may represent pathogenic T cell clones involved in the initiation of MS.

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