Direct Ex Vivo Analysis of Activated, Fas-sensitive Autoreactive T Cells in Human Autoimmune Disease

By Katarzyna D. Bieganowska, Lara J. Ausubel, Yalda Modabber, Elissa Slovik, Wells Messersmith, and David A. Hafler

From the Laboratory of Molecular Immunology, Center for Neurologic Diseases, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts 02115

Summary

The frequency of clonally expanded and persistent T cells recognizing the immunodominant autoantigenic peptide of myelin basic protein (MBP)p85-99 was directly measured ex vivo in subjects with typical relapsing remitting multiple sclerosis (MS). T cells expressing mRNA transcripts encoding T cell receptor (TCR)-α and -β chains found in T cell clones previously isolated from these subjects recognizing the MBPp85-99 epitope were examined. In contrast to frequencies of 1 in 10^5–10^6 as measured by limiting dilution analysis, estimates of the T cell frequencies expressing MBPp85-99–associated TCR chain transcripts were as high as 1 in 300. These high frequencies were confirmed by performing PCR on single T cells isolated by flow cytometry. MBPp85-99 TCR transcripts were present in IL-2 receptor α–positive T cells which were induced to undergo Fas-mediated cell death upon antigen stimulation. These data demonstrate that at least a subpopulation of patients with MS can have a very high frequency of activated autoreactive T cells.

M ultiple sclerosis (MS)[1] is a chronic inflammatory disease characterized by lymphocytic infiltration and demyelination in the central nervous system (CNS) thought to be initiated by activated T cells recognizing myelin components of the CNS (1–5). T cells with high affinity receptors recognizing myelin basic protein (MBP) and proteolipid protein (PLP) are part of the normal T cell repertoire and are present in the blood of MS patients as well as in healthy individuals with comparable frequencies of 1 in $\sim$$10^5$–$10^6$ T cells, as revealed by limiting dilution analysis (LDA; 6–8). However, determination of the frequency of antigen-specific T cells in LDA assays is based upon the ability of these cells to proliferate in response to antigen. Thus, estimated frequencies are confounded by the need to grow short term T cell lines and do not allow detection of antigen-specific T cells that respond to antigen by means of cytokine production in the absence of proliferation (9). Moreover, investigations using cloning techniques that preferentially allow the growth of activated T cells have suggested that autoreactive T cells from MS patients are activated in vivo as compared to the autoreactive T cells from normal individuals, and that the precursor frequencies of in vivo activated T cells responding to MBP or PLP are in fact higher in MS patients (10, 11). Thus, different T cell cloning strategies may influence the calculated frequency of autoreactive T cells.

The MBPp85-99 epitope is one of the immunodominant epitopes of MBP (6, 7, 12). We have previously determined the TCR sequences of clonally persistent MBPp85-99–reactive T cells both in patients with MS and in normal individuals (13). This enabled us to develop methods to directly estimate the frequency of MBPp85-99–reactive T cells by measuring mRNA transcripts encoding the TCR-α and -β chains ex vivo in peripheral blood without in vitro manipulation. Moreover, the ability to directly measure frequencies of MBPp85-99–reactive T cells allowed us to functionally examine the response of autoreactive T cells to antigen.

In contrast to frequencies of one in 10^5 to 10^6 as measured by LDA, estimates of the T cell frequencies expressing MBPp85-99 associated TCR chain transcripts were as high as 1 in 300. MBPp85-99–associated TCR transcripts were present in IL-2 receptor α (IL-2Rα)–positive T cells which were induced to undergo Fas-mediated cell death upon antigen stimulation. These data demonstrate that measurements of T cell frequencies by short-term T cell cloning and thymidine incorporation, as is used by LDA, do not allow for correct estimates of activated antigen-reactive T cells. Additionally, at least a subpopulation of patients with

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[1] *Abbreviations used in this paper:* CDR3, third-complementarity-determining region; CNS, central nervous system; dd, double distilled; dNTP, deoxynucleotide triphosphate; dT, deoxythimidine; IL-2Rα, IL-2 receptor α; LB, luria broth; LDA, limiting dilution analysis; MBP, myelin basic protein; MS, multiple sclerosis; PLP, proteolipid protein; WMNC, whole mononuclear cells.

MS can have a very high frequency of activated autoreactive T cells.

Materials and Methods

MBPp85-99–reactive T Cell Clones. Investigations were approved by the human subjects committee of the Brigham and Women's Hospital (Boston, MA). MBPp85-99–reactive clones from the subjects (two patients with relapsing remitting MS and two normal control subjects) were established previously and T cell receptor sequences published (13). In brief, amino acid TCR-α and -β chain junctional region sequences were as follows: patient Ob, clone Ob.2F3 Vα3.1-TDATSGTYKYIFGTGTRLKVLA-Cα, Vβ2.1-RDLTSGSLNEQFFGPGTRLTVL-Cβ; patient Hy, clone Hy.1G11 Vα3.1-TDTGGSYIPTFGRGTSLIVHP-Cα, Vβ17.1-TSGSYNEQFFGPGTRLTVL-Cβ, clone Hy.2B6 Vα3.1-TDA-GGQNFVFGPGTRLSVLP-Cα, Vβ17.1-TDWSSYNEQFFGPG-TRLTVL-Cβ, clone Hy.2E11 Vα3.1-TDSGGSYIPTFGRGT-SLIVHP-Cα, Vβ4-PSGQGTYGYTFGSGTRLTVV-Cβ; control Nb, clone Nb17.8 Vα8-ASISDDMRFGAGTRLTVKP-Cα, Vβ12-YSPLGNEQFFGPGTRLTVL-Cβ; control Jl, clone NSJl5 Vα18-SGYNNNDMRFGAGTRLTVKP-Cα, Vβ21-LTVGSYNEQ-FGPGTRLTVL-Cβ, clone NSJl 14.5 Vα18-SGSNDYKLSFG-AGTTVTVRA-Cα, Vβ14-SSIPGQPQHFGDGTRLSIL-Cβ. The third complementarity-determing region (CDR3) probes were named according to the first three amino acids in the NH₂-terminal sequence of the junctional region.

PCR Amplification of TCR Chains. mRNA extractions were performed using the RNAzol B method (Teltest, Inc., Friendswood, TX). RNA was coprecipitated with 5 μg of tRNA (Sigma Chemical Co., St. Louis, MO) in isopropanol overnight at −20°C. After washing with 70% ethanol, pellets were air dried and resuspended in double distilled (dd) H₂O. First strand cDNA synthesis was primed with oligo(deoxythimidine; dT) in 11 μl reaction and the samples heated to 70°C for 10 min. 4 μl of 5× buffer, 2 μl 0.1M dithiothreithiol, and 1 μl each of 10 mM deoxynucleotide triphosphate (dNTPs), 33 U of RNAsin, and 200 U of moloney murine leukemia virus reverse transcriptase (all from Promega Corp., Madison, WI) were then added. cDNA synthesis was carried out at 42°C for 60 min, and ddH₂O was added to a final volume of 200 μl. 10 μl was used for each PCR. 50-μl PCR reactions contained 0.25 μg of forward and reverse primer, 1U of Taq polymerase, and 20 μl of a mix containing dNTPs and Taq buffer (Perkin-Elmer Corp., Branchburg, NJ). Amplifications were done for 35 cycles by using the following temperature profile: 94°C denaturation for 1 min, 60°C annealing for 2 min, and 72°C extension for 3 min with a final extension step at 72°C for 10 min. Sequences of primers were: Vα3, 5′ - GGA GTG TCT TTG GTG ATT CTA TGG CTT CAA - 3′; Vα8, 5′ - CGA GCT TTA TTT ATG TAC TTG TGG CTG CAG - 3′; Vα18, 5′ - TGT CAG GCA ATG ACA AGG GAA GCA ACA AAG - 3′; Cα reverse primer, 5′ - TTG TTG CTC CAG GCC ACA GCA CTG TTG CTC - 3′; Vβ17.1, 5′ - TTT CAG AAA GGA GAT ATA GCT GAA GGG TAC - 3′; Cβ reverse primer, 5′- GGC AGA CAG GAC CCC TTG CTG GTA GGA CAC -3′; Cβ internal primer: 5′- TGT GCA CCT CCT TCC CAT TCA CCC ACC AGC - 3′; Amplified products were analyzed on 1% agarose gels stained with ethidium bromide.

Colony Hybridization. PCR products were purified using PCR purification system (Promega Corp.). Purified PCR reactions were ligated into pCRII vectors (TA cloning system; Invitrogen, San Diego, CA) in the presence of T4 ligase by incubation at

14°C overnight. 50 μl of competent bacteria (INVαF′; Invitrogen) were then transformed with ligation products and screened for inserts on X-galactosidase–ampicillin containing luria broth (LB) agar medium (GIBCO BRL, Gaithersburg, MD). After overnight culture at 37°C, white colonies were transferred into 96-well flat-bottom plates containing 200 μl LB medium with 50 mg/liter of ampicillin. Plates were incubated for an additional 18 h at 37°C and several replicas of each plate were made. DNA was bound to nitrocellulose (GIBCO BRL) by standard procedures followed by hybridization with the appropriate Vα or CDR3 region–specific probes. Oligonucleotide probes were endlabeled with the use of γ-³²ATP and T4 polynucleotide kinase. Hybridizations were performed for 18 h at 37°C in a buffer containing 6 × SSC/0.05% pyrophosphate/5× Denhardt/0.1mg/ml of denatured salmon sperm DNA. After hybridizations filters were washed with 6 × SSC/0.05% pyrophosphate at 55–65°C and exposed on Kodak film. TCR-α CDR3 probe sequences were: patient Ob, Ob-TDA 5′ - ACG GAC GCA ACC TCA GGA ACC TAC AAA TAC - 3′; patient Hy, Hy-TDA 5′ - ACG GAC GCA GGA GGT CAG AAT TTT GTC TTT - 3′, Hy-TDT 5′ - ACG GAT ACA GGA GGA AGC TAC ATA CCT ACC - 3′, Hy-TDS 5′ - GCT ACG GAC TCA GGA GGA AGC TAC ATA - 3′; control Jl, Jl-SSI 5′ - CTG AGT TCA ATT ATG GTG GTG CTA CA - 3′, Jl-SGS 5′ - G GCT CTG AGT GGT TCT AAC GAC - 3′; control Nb, Nb-ASI 5′ - TGT GCA GCA AGT ATT AGT GAT GAC A - 3′. TCR-β CDR3 probe sequences were: patient Hy, Hy-TDW 5′ - ACT GAC TGG AGC TCC TAC AAT GAG CA - 3′, Hy-TSG 5′ - ACT AGC GGC TCC TAC AAT GAA CAG TTC TT - 3′.

Specificity of Junctional Region Probes. The probes bound exclusively to the sequences present in the original T cell clones. cDNA from the original T cell clones were used to examine probe hybridization conditions. The T cell clones Ob.1A12 and Ob.2F3 from patient Ob differ by only three nucleotides in the N region (one amino acid), sharing the same Vα3.1 and Jα40 TCRs (4). The probe specific for clone Ob.2F3 (Ob-TDA probe) hybridized to Vα3.1 amplified cDNA from that T cell clone, but did not crosshybridize to Ob.1A12, thus demonstrating the probe's specificity. For patient Hy, two CDR3 region probes were designed; one hybridized exclusively to the CDR3 region with an Nα-Jα region beginning with TDA (Hy-TDA probe), whereas the other probe was designed to hybridize to the CDR3 region with an Nα-Jα region beginning with TDT (Hy-TDT probe). This latter probe crosshybridized to the CDR3 region from another MBP reactive T cell clone sequence with an Nα-Jα region beginning with TDS, which was a less frequently observed MBP-reactive T cell clone in this patient. The CDR3 probes were specific for each patient as the probe from patient Ob did not hybridize with T cells stimulated with MBPp85-99 from patient Hy and vice versa (data not shown). The two Jl-SSI and Jl-SGS probes designed for identification of the different Vα18-bearing clones of control Jl did not crosshybridize, and the Nb-ASI probe for control Nb MBPp85-99–reactive T cell clones similarly hybridized with the appropriate TCR-α chain.

DNA Sequence Analysis. Bacterial colonies were expanded by overnight culture in 3 ml of LB-ampicillin medium. Plasmids were isolated using Magic minipreps as described by the manufacturer (Promega Corp.). Double stranded DNA was sequenced using the sequenase protocol (U.S. Biochem. Corp., Cleveland, OH) with [³⁵S]dATP as a radioactive tracer and the internal primer: 5′ - CTT GTC ACT GGA TTT AGA GTC TCT CAG CTG - 3′ for TCR-α chain and 5′ - TGT GCA CCT CCT TCC CAT TCA CCC ACC AGC - 3′ for TCR-β chain.

Culture of Whole Mononuclear Cells. Whole mononuclear cells (WMNC) were separated by a Ficoll gradient centrifugation, and 10^6 cells were incubated in 24-well plates with either native peptide MBPp85-99 (amino acid sequence ENPVVHFFKNIVTPR, 93K) or MBPp85-99 with amino acid substitutions at position 93 (93L, 93A, 93R, peptides synthesized by Biopolymer Laboratory, Harvard Medical School) at a final concentration 10 μM, anti-CD3 mAb (OKT3, 1:1,000), or no stimuli in growth medium (RPMI 1640 medium supplemented with 10% autologous serum, 2 mM l-glutamine, 10 mM Hepes 100 U/100 μg/ml penicillin/strepto-mycin; all from BioWhittaker Inc., Walkersville, MD). After 7 d, cells cultured with MBP peptides were restimulated with 10^6 antigen-pulsed autologous blood WMNC prepared by incubating autologous antigen-presenting cells with the appropriate peptide for 2 h followed by three washes in medium and irradiation (5,000 rads). On day 9, 10% IL-2 (Human T-Stim; Collaborative Biomedical Products, Bedford, MA) –containing medium was added to each tube. On day 14, the cultures were harvested and mRNA was extracted.

For estimating antigen-induced apoptosis, WMNC were cultured for 72 h with 0, 0.5, 5, or 50 μM MBPp85-99 either with control antibody alone (1,000 ng/ml isotype control antibody), or with 500 or 1,000 ng/ml of anti-CD95 mAb (clone ZB4; Immunotech, supplied by Coulter Immunology, Hialeah, FL).

Cell Staining and Sorting. WMNC were incubated with mouse anti–TCR Vβ17.1 chain mAb (clone E17.5F3; Immunotech, Westbrook, ME) for 30 min at 4°C. Indirect staining was followed by incubation with goat anti–mouse IgG and IgM Fab′ fragments conjugated with FITC (Tago Immunologicals, Camarillo, CA). Anti-CD3 mAb and mouse IgG (both a gift from Coulter Corp., Miami, FL) were used as positive and negative controls. Vβ17.1-positive and -negative populations were sorted on a Coulter Sorter (type EPICS). For sorting IL-2Rα-positive and IL-2Rα-negative T cell populations, WMNC were stained with FITC-conjugated anti–IL-2Rα mAb (Coulter Corp.).

Single-sided PCR Amplification. The RNA pellet was resuspended in 18 μl of water and annealed with 15 μl oligo(dT) for 10 min at 70°C. cDNA synthesis was performed in a reaction containing 12 μl of 5× buffer, 6 μl of 0.1 M dithiothreithiol, 3 μl of RNAsin, 5 μl of reverse transcriptase (all from Promega), and 3 μl of dNTPs (Pharmacia, Uppsala, Sweden) for 1 h at 42°C. cDNA was precipitated with 1/10 volume of 3 M ammonium acetate and 2 volumes of ethanol at −70°C. The cDNA pellet was washed in 70% ethanol and air dried. Aliquots of cDNA were homopolymer tailed with terminal deoxynucleotidyltransferase and deoxycytosine triphosphate. Second strand synthesis was carried out using Taq polymerase and an oligo-(dG) primer (5′ - GATAGT-CGACGGGGGGGGGG - 3′).

Single Cell PCR. Single TCR-Vβ17.1–expressing cells were directly sorted onto V-bottom 96-well plates containing 150 μl of PBS. Cells were then centrifuged and 5 μl of ddH$_2$O was added to each well followed by boiling for 5 min. First strand cDNA synthesis was performed as described. The entire cDNA reaction was used for the first 35 cycles of PCR with the Vα3.1-specific primer together with the Cα-specific primer. 2 μl of amplification reaction was reamplified for the additional 35 cycles of PCR with an internal Cα primer (5′ - CTT GTC ACT GGA TTT AGA GTC TCT CAG CTG - 3′) and the same Vα3.1-specific primer.

Spiking Experiment. Increasing numbers of the T cell clone Hy1G11 were spiked into 500,000 WMNC from peripheral blood of subject Ob resuspended in 1.0 ml of RPMI. The mRNA was extracted and the frequency of Vα3 transformants hybridizing to the Hy-TDT probe was measured as described above. The

frequency of Vα3-positive T cells was measured by anchor PCR as described above. The expected versus the measured frequency of T cells expressing the Hy CDR3-TDT were plotted.

Results

Detection of Junctional Region Sequences Associated with MBPp85-99 Recognition in Peripheral Blood T Cells. We analyzed the TCR-α chain sequences of MBPp85-99–reactive T cell clones isolated from the MS patients and normal subjects. The MS patients chosen were those previously shown to have clonally expanded and persistent MBPp85-99–reactive T cells. The controls chosen had equal frequencies of MBPp85-99–reactive T cell clones, as measured by LDA (13). The MBPp85-99–reactive T cell clones studied from the MS patients used Vα3.1 chains, whereas Vα18 and Vα8 chains were used in the T cell clones from the controls. We measured the frequency of TCR-α sequences associated with MBPp85-99–reactive T cells directly in the peripheral blood by PCR amplification of TCR-α chains followed by subcloning and colony hybridization analysis. Over 10,000 TCR-α transformants were screened for binding of the Vα- and CDR3-specific probes. Probes were designed to bind the CDR3 coding regions of the TCR-α chains under stringent hybridization conditions, and the specificites of the probes were confirmed on the original T cell clones. The CDR3 region probes were named according to the first three amino acids in the NH$_2$-terminal sequence of the junctional region.

Using this approach, we could identify TCR-Vα chains expressed in MBPp85-99–reactive T cells in MS patients (Table 1, Fig. 1 *A*). Specifically, the percentage of Vα3.1-positive transformants hybridizing with the Ob-TDA probe was 0.8% of Vα3.1 chains expressed in patient Ob; the percentages were 1.6% for probe Hy-TDA and 2.4% for probe Hy-TDT of Vα3.1 chains expressed in patient Hy (Table 1). Repeated experiments measuring the percentage of transformants hybridizing with either probe over a two-yr time interval yielded similar frequencies (Table 1). As expected, there was no crosshybridization of Hy probes with Ob transformants or of Ob probes with Hy transformants. The sequencing of 20 transformants expressing a TCR-α chain that hybridized to the Ob-TDA probe in patient Ob and 25 transformants that hybridized to either the Hy-TDA or Hy-TDT probes in patient Hy, demonstrated the same TCR-α sequence as that expressed in the original MBP-reactive T cell clones. As expected, DNA from 20 random transformants that did not hybridize to the CDR3 probes contained different TCR-α junctional region sequences. In control subjects, after screening TCR-α transformants with Jl-SSI and Jl-SGS probes for Jl and Nb-ASI probe for Nb, we were unable to detect any sequences associated with recognition of MBPp85-99 in peripheral blood T cells (Table 1).

PCR Analysis of TCR Can Specifically Measure Clonal Expansion of Antigen-specific T Cells. It was important to show that the assay could specifically detect antigen-induced clonal expansion of T cells. This necessarily required in vitro rather than in vivo experiments where WMNC were

Table 1. *Frequency of CDR3-specific Sequences Associated with MBPp85-99 Recognition in Unstimulated Peripheral Blood Lymphocytes of MS Patients and Controls*

Patient Ob			
Vα3.1 transformants	Ob-TDA* transformants		
1,173	9 (0.8%)		

Patient Hy			
Experiment	Vα3.1 transformants	Hy-TDA* transformants	Hy-TDT* transformants
1	252	4 (1.6%)	6 (2.4%)
2	162	5 (3.1%)	10 (6.2%)
3	126	2 (1.6%)	2 (1.6%)
4	128	5 (3.9%)	2 (1.6%)

Control Jl		
Vα18 transformants	Jl-SSI transformants	Jl-SGS transformants
275	0	0

Control Nb	
Vα8 transformants	Nb-ASI transformants
313	0

cDNA from unstimulated peripheral blood T cells from each subject were amplified with their respective Vα and Cα primers, and the purified PCR products ligated into pCRII vectors were used to transform competent bacteria. Transformants were transferred to 96-well plates containing medium and allowed to expand. Replicas of each transformed colony were screened for binding to the respective TCR-Vα chain and the CDR3 region probe associated with TCR-Vα chain originating from an MBPp85-99–reactive T cell of that subject. *Total of 19 transformants hybridizing to the correct CDR3 region were directly sequenced to confirm the correct identity of the TCR chain.

stimulated either nonspecifically by cross-linking the TCR with anti-CD3 mAb or with the specific antigen MBPp85-99. 14 d after stimulation with MBPp85-99, the percentage of TCR-Vα3.1 transformants expressing junctional region sequences present in the specific MBPp85-99–reactive T cell clones studied went from 0.8 to 90.2% in patient Ob and from a total of 4.0 to 86.4% in patient Hy for Hy-TDA and Hy-TDT sequences combined (Fig. 1 *B* and Table 2 *A*). This increase was antigen specific as it was not seen upon antibody-mediated CD3 cross-linking. In contrast, none of the previously observed TCR-α sequences expressed in MBPp85-99–reactive T cell clones were found in controls Nb and Jl.

A further control was performed to demonstrate the assay's specificity and sensitivity. WMNC were stimulated with either MBPp85-99 or with analogue peptides substituted at position 93, a TCR contact residue. We found that while stimulation of WMNC with the native peptide induced marked increases in clonal expansion of the T cells as measured by the assay, stimulation of WMNC with MBPp85-99 with a single amino acid substitution markedly diminishes this expansion (Table 2 *B*). Interestingly, these data with PCR amplification and colony hybridization of mRNA isolated after stimulation of WMNC with the analogue peptides reflects experiments with in vitro culture of WMNC with analogue peptides followed by T cell cloning. That is, T cell clones generated with MBPp85-99 stimulation cross-reacted with MBPp85-99 (93K→R) and (93K→L) peptides, but not (93K→A) peptides (14). Furthermore, TCR sequences of the T cell clones that were found to be cross-reactive with the MBPp85-99(93R) and MBPp85-99(93L) peptides used the Hy-TDS sequence that was also detected in this assay using PCR amplification, followed by colony hybridization. In total, these data demonstrate the very high specificity of this assay in detecting antigen-specific clonal expansion of peripheral blood T cells.

Estimation of the Total Frequency of MBPp85-99–Reactive T Cells in Peripheral Blood. Assuming that each T cell expressing Vα3.1 in the peripheral blood contributes equally to the PCR amplification product using the Vα3.1-Cα primer pairs, the frequency of transformants with the TCR-α sequence associated with MBPp85-99 reactivity should reflect the frequency of circulating T cells expressing that TCR-α
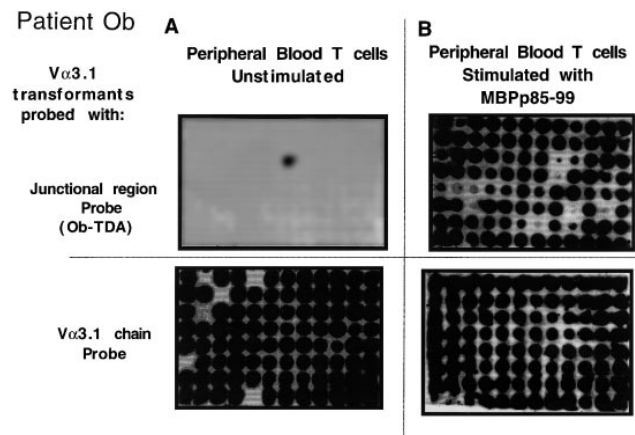

Figure 1. Frequency of TCR-Vα3.1 transformants expressing the CDR3 region sequence present in an MBPp85-99–reactive T cell clone in MS patient Ob. (*A*) A representative experiment is shown, using mRNA from peripheral blood lymphocytes. cDNA was synthesized and amplified with Vα3.1- and Cα-specific primers. PCR products were ligated into pCRII vectors and competent *Escherichia coli* were transformed with ligation products. Transformants were grown in 96-well plates and were transferred to nitrocellulose paper in duplicates. Blots were hybridized with either Vα3.1 probe (*bottom*) or a specific Ob-TDA probe recognizing TCR-Vα3.1 junctional region sequence expressed in a previously isolated MBP-reactive T cell clone (*top*). (*B*) The same peripheral blood lymphocytes from subject Ob were stimulated with MBPp85-99 for 7 d, followed by restimulation with antigen-pulsed WMNC and, on day 9, the addition of IL-2. On day 14, mRNA was extracted from the antigen-stimulated T cells and the proportion of transformants hybridizing with the Ob-TDA–specific probe after Vα3.1 chain amplification was measured.

Table 2. *Frequency of CDR3-specific Sequences Associated with MBP Recognition After 14 d Stimulation of WMNC*

A Patient Ob	Vα3.1 transformants	Ob-TDA transformants*		
Day 0 (unstimulated)	1173	9 (0.8%)		
Day 14:				
no antigen	118	0		
MBPp85-99	164	148 (90.2%)		
anti-CD3	84	0		

Patient Hy	Vα3.1 transformants	Hy-TDA* transformants	Hy-TDT* transformants	Hy-TDS* transformants
Day 0 (unstimulated)	252	4 (1.6%)	6 (2.4%)	–
Day 14:				
no antigen	68	0	0	–
MPPp85-99	513	241 (47.0%)	202 (39.4%)	–
anti-CD3	94	7 (7.4%)	0	–
B				
Day 0 (unstimulated)	126	2 (1.6%)	2 (1.6%)	0
Day 14:				
MBPp85-99	91	43 (47.2%)	13 (14.3%))	1 (1.1%)
p85-99 (93L)	89	5 (5.6%)	2 (2.2%)	3 (3.4%)
p85-99 (93A)	92	1 (1.1%)	0	0
p85-99 (93R)	74	1 (1.3%)	0	7 (9.4%)

Stimulation of WMNC with (*A*) MBPp85-99, or anti-CD3 mAb and (*B*) MPBp85-99 peptides with substitutions at the TCR contact residue at position 93. WMNC were separated by a Ficoll gradient centrifugation, and 10^6 cells were incubated in 24-well plates with either native peptide MBPp85-99 (amino acid sequence ENPVVHFFKNIVTPR, 93K) or MBPp85-99 with amino acid substitutions at position 93 (93L, 93A, 93R; peptides synthesized by Biopolymer Laboratory, Harvard Medical School) at final concentration 10 μM, anti-CD3 mAb (OKT3, 1:1000), or no stimuli in growth medium. On day 14, the cultures were harvested and mRNA was extracted. *Specificity of probe's binding was verified by sequencing 11 of Ob-TDA–, 5 of Hy-TDA–, 5 of HY-TDT–, and 7 of Hy-TDS–positive transformants.

chain. To estimate the frequency of all T cells with the TCR-α chain expressed in MBPp85-99–reactive T cells, it was necessary to determine the proportion of T cells using Vα3.1 among all Vα chains expressed. This was done by amplifying TCR-α transcripts from WMNC using a modification of the rapid amplification of cDNA ends and anchored PCR methods. The percentage of Vα3.1 chains among all TCR-α chains in unstimulated WMNC was 5.1% in patient Ob and 8.1% in patient Hy. The frequency of circulating MBP-reactive T cells in unstimulated WMNC was estimated by multiplying the frequency of Vα3.1 among all Vα chains by the frequency of specific CDR3 sequences expressed in the amplified TCR-Vα3.1 chains associated with recognition of MBPp85-99 (Table 1). Thus, the estimated frequency of T cells recognizing MBPp85-99 in unstimulated WMNC of patient Ob was 3.9×10^{-4} and in patient Hy 3.2×10^{-3} (1.3×10^{-3} for the Hy-TDA sequence, and 1.9×10^{-3} for the Hy-TDT sequence).

Pairing of TCR-α and -β Chains on T Cells Specific for MBPp85-99. A series of experiments were performed to determine whether expanded clonotypes bearing Hy-TDA or Hy-TDT sequences are paired exclusively with Vα3.1 and Vβ17.1 chains as in the original MBPp85-99 reactive clones. First, WMNC cultured for 14 d with MBPp85-99 were sorted into Vβ17.1-positive and Vβ17.1-negative populations, and examined for expression of Vα3.1-Hy-TDA or Hy-TDT sequences. The same frequencies of Hy-TDA and Hy-TDT sequences in the Vβ17.1-positive population (45.5% for TDA and 47.3% for TDT) and the unsorted population were observed, while there were no Hy-TDA– or Hy-TDT–detectable sequences in the Vβ17.1-negative population. These results indicated that after antigen stimulation, TCR-Vα3.1 chain Hy-TDA and Hy-TDT sequences associated with MBPp85-99 reactivity are paired only with Vβ17.1 chains. Secondly, in the experiments using anchor PCR in which all Vα chains were amplified, CDR3 probes recognizing sequences present in the TCR-Vα3.1 chains of MBP reactive T cell clones from both patients Hy and Ob did not hybridize with transformants that expressed different Vα chains (data not shown), confirming that the

CDR3 sequences are only associated with the Vα3.1 chains. Lastly, the definitive experiment to prove correct pairing of TCR-α and -β chains associated with MBPp85-99 reactivity before antigen stimulation required PCR amplification of both TCR-α and -β chains from T cells isolated directly from peripheral blood at limiting dilution. Our attempts to simultaneously amplify Vβ17.1 chains from the same single cell expressing Vα3.1 Hy-TDA and Hy-TDT sequences were unsuccessful due to the lower efficiency of the Vβ17.1-Cβ PCR despite multiple attempts to increase the efficiency of the amplification procedure. However, this analysis was successfully performed on Vβ17.1-positive cells sorted by flow cytometry at 10 cells/well where the corresponding TCR-β chain sequence identified in the previously isolated MBP reactive T cell clones (Vβ17.1-TSG sequence identified in clone Hy.1G11) was found with the Vα3.1 Hy-TDT sequence in the same well. In total, these data strongly suggest that there is predominantly correct pairing of TCR-α and -β chains associated with MBP-reactive T cells isolated directly from peripheral blood.

Single Cell PCR of TCR-α Chain Sequences. A second approach was used to confirm the high frequencies of MBP-p85-99–reactive T cells circulating in blood from subjects with MS. Single T cells expressing Vβ17.1 were sorted by flow cytometry directly into single wells. PCR using seminested primers for the Vα3.1 chains followed by probing with Hy-TDA– and Hy-TDT–labeled probes was performed on each individual mRNA sample extracted from a single T cell. Out of a total of 192 wells with single Vβ17-positive T cells that were sorted by flow cytometry, 161 gave an appropriate PCR product. 3 of the 161 single cells analyzed hybridized to the Hy-TDA probe and 1 hybridized to the Hy-TDT probe (Table 3). The use of the correct TCR-α chain in the Hy-TDT– or Hy-TDA–positive transformants was confirmed by sequencing. As 5.3% of the T cells expressed Vβ17.1 as measured by flow cytometry, the frequency of T cells expressing Vβ17.1 chains and TCR-α chain sequences found in MBPp85-99–reactive T cells was calculated to be 1.3×10^{-3} (for Hy-TDA and Hy-TDT sequences combined), comparable to the 3.2×10^{-3} calculated by examination of WMNC by PCR and colony hybridization (Table 4). In total, these data confirm the high frequency of circulating MBPp85-99–reactive T cells and exclude the possibility that this was secondary to increased amounts of TCR mRNA transcripts in activated MBPp85-99–reactive T cells or to preferential amplification of the particular Vα chain.

Spiking Experiment. A third approach where MBP-reactive T cells were spiked into peripheral blood T cells from another subject was used to confirm the high frequencies of MBP-reactive T cells observed in the blood. Increasing numbers of the T cell clone Hy1G11 were spiked into 500,000 WMNC from peripheral blood of subject Ob, mRNA was extracted, and the frequency of Vα3 transformants hybridizing to the Hy-TDT probe measured. The frequency of Vα3-positive T cells measured by anchor PCR were multiplied by the percent of transformants that hybridized to the Hy-TDT probe. A total of 795 Vα3-positive transfor-

mants were analyzed at predicted frequencies between 2×10^{-6} and 2×10^{-2}. The expected versus the measured frequency of T cells expressing the Hy CDR3-TDT were plotted (Fig. 2). At predicted frequencies of 2×10^{-5}, there was no detectable hybridization to the 133 Vα3 transformants examined. This likely represents the lower limit of detection of the assay with examination of ~125 transformants. The assay was less precise at a predicted frequency of 2×10^{-4} where sampling errors may occur; in this experiment, there were 2 of 187 positive transformants. Although at very high numbers of spiked T cell clones, the assay may have slightly underestimated the frequency of MBP-reactive T cells, at predicted frequencies of 2×10^{-3} MBP-reactive T cells, which we observed in peripheral blood of MS patients, the measured frequency in the spiking assay was in close agreement (1.12×10^{-3}).

Fas-mediated Activation Induced Cell Death of IL-2Rα+ MBP-reactive T Cells There was an ~1,000-fold higher frequency of MBP-reactive T cells calculated by direct PCR and colony hybridization as compared to LDA and these data are summarized in Table 4. The high frequency of MBP-reactive T cells in the peripheral blood of the patients with MS as compared to the normal individuals was puzzling considering that the frequency of T cells as calculated by LDA was similar. These data suggested that the frequency of MBP-reactive T cells as calculated by LDA was accurate in the normal subjects, but may have been grossly underestimated in the patients with MS. On the basis of findings that activated cells are more prone to antigen-induced cell death (15), we hypothesized that subpopulations of autoreactive T cells in patients with MS may express IL-2Rα, and thus may undergo apoptosis in LDA conditions leading to a lower calculated frequency. In this regard, Pelfrey et al. have demonstrated that MBP-reactive T cell lines from patients with MS are highly susceptible to activation-induced cell death (16). The activation state of MBPp85-99–reactive T cells could be examined by measuring the frequency of TCR-Vα3.1 transformants obtained from IL-2Rα–positive and –negative populations that hybridized to either the Hy-TDA or Hy-TDT probes. We measured the distribution of Hy-TDA and Hy-TDT clonotypes in IL-2Rα–positive and –negative populations on two different time points, 3 mo apart. On the first time point

Table 3. *Single Cell PCR*

Sorted populations	No. of transformants expressing TCR-α (Hy-TDA) sequence	No. of transformants expressing TCR-α (Hy-TDT) sequence
Vβ17.1 positive	3/161	1/161

Single T cells expressing Vβ17.1 were sorted directly into 96-well plates. PCR using seminested primers were performed on 161 wells. Each well containing a PCR amplification product that hybridized to the Hy-TDA or Hy-TDT probes was found to have a correct sequence.

Table 4. *Frequency of Circulating MBP-reactive T Cells in Patient Hy as Determined by Different Methodologies*

Methodology	Estimated frequency	Calculation
PCR and colony hybridization of WMNC	3.2×10^{-3}*	(Frequency of TCR-Vα3.1 transformants calculated by anchor PCR) $\times$ (frequency of TCR-Vα3.1 transformants expressing Hy-TDA and Hy-TDT sequences associated with MBPp85-99 reactivity)
PCR and colony hybridization of single Vβ17.1-expressing T cells sorted by flow cytometry	1.3×10^{-3}*	(Frequency of Vβ17.1-expressing T cells calculated by flow cytometry) $\times$ (frequency of single Vβ17.1-expressing T cells coexpressing TCR-Vα3.1 Hy-TDA or TCR-Vα3.1 Hy-TDT sequences associated with MBPp85-99 reactivity)
Limiting dilution analysis of WMNC stimulated with MBP	2.3×10^{-6}‡	Poisson frequency estimation

*Frequency of MBPp85-99–reactive T cells expressing Vα3.1–Hy-TDA and Vα3.1–Hy-TDT sequences.
‡Frequency of MBP-reactive T cells.

tested, we found increased frequency of Hy-TDA sequence in IL-2Rα–positive population, whereas on the second time point we could not detect any Vα3.1 transformants expressing Hy-TDA sequence. In contrast, there was an equal distribution of Vα3.1 Hy-TDT sequence among IL-2Rα–positive and –negative populations on the two time points tested (Table 5).

To determine whether self antigen could induce selective loss of autoreactive T cells, WMNC were cultured with increasing concentrations of MBPp85-99 peptide and the frequency of TCR-Vα3.1 transformants hybridizing to either the Hy-TDA or Hy-TDT probes was measured before and after 72 h of culture. Note that the measurement of Hy-CDR3 frequencies before incubation with MBP were performed from three separate cultures and represent both IL-2R–positive and –negative populations. There was an

Figure 2. Increasing numbers of the T cell clone Hy1G11 were spiked into 500,000 WMNC from peripheral blood of subject Ob. The frequency of Vα3 transformants hybridizing to the Hy-TDT probe was measured and the expected versus the measured frequency of T cells expressing the Hy CDR3-TDT were plotted.

almost total loss of transformants expressing the Hy-TDT sequence, whereas no changes were observed in transformants expressing the Hy-TDA sequence (Fig. 3, *A* and *B*). Interestingly, as described above, at this time point, Hy-TDA sequence was only found in IL-2Rα–negative population. Since it has been demonstrated that antigen stimulation of activated T cells expressing IL-2Rα induces apoptosis mediated by expression of Fas (CD95) and Fas ligand on the T cell surface (17–24), we examined whether antigen stimulation of peripheral blood T cells in the presence of blocking anti-CD95 mAb selectively inhibited the loss of TCR-Vα3.1 Hy-TDT–expressing T cells. As shown in Fig. 3 *B*, anti-CD95 mAbs totally blocked the MBPp85-99–induced loss of transformants hybridizing to Hy-TDT probe while having no effect on the frequency of Hy-TDA transformants. As T cells with the TCR-Vα3.1–Hy-TDT sequence expressed IL-2Rα, these data indicate that the low frequency of MBPp85-99–reactive T cells as measured by LDA was partly due to Fas-mediated apoptosis. The initiation of immunotherapy that altered the frequency of MBP-reactive T cells precluded this analysis of activated T cells in subject Ob.

Discussion

We measured the frequency of clonally expanded and persistent T cells recognizing the immunodominant MBPp85-99 epitope in subjects with typical relapsing remitting MS. Single T cells expressing mRNA transcripts encoding TCR-α and -β chains found in T cell clones previously isolated from these subjects recognizing the MBPp85-99 epitope were examined. In contrast to frequencies of 1 in 10^5 to 10^6 as measured by LDA, estimates of the T cell frequencies expressing TCR chain transcripts associated with MBPp85-99 recognition were as high as 1 in 300.

In retrospect, the high frequencies of MBPp85-99–reactive T cells with presumed chronic stimulation is perhaps not surprising. Subjects with HTLV-I and HIV infection have
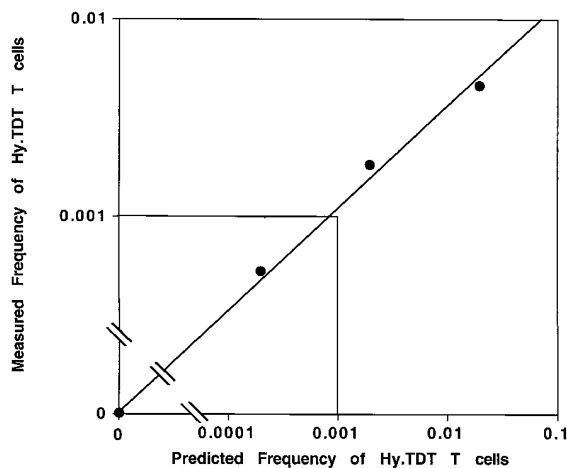
Table 5. *Distribution of Hy-TDT and Hy-TDA Sequences Associated with MBP Recognition in IL-2Rα–negative and –positive Populations*

	First time point		Second time point	
Probe	IL-2Rα⁻	IL-2Rα⁺	IL-2Rα⁻	IL-2Rα⁺
	%		*%*	
CDR3-TDA	1.7	8.1	3.1	0
CDR3-TDT	3.4	3.0	6.6	4.7

IL-2Rα–negative and –positive populations were sorted on two time points, 3 mo apart. WMNC were stained with FITC-conjugated anti–IL2Rα mAb (Coulter Corp.) and IL-2Rα–positive and –negative T cell populations were sorted. cDNA synthesis, PCR, and colony hybridization were performed as described. A total of 1,152 TCR Vα3.1 positive transformants were analyzed for binding of Hy-TDA and Hy-TDT junctional region probes.

high frequencies of virus reactive T cells as measured ex vivo in peripheral blood using direct cytotoxicity assays (25–27). In contrast, the LDA analysis of CTL frequencies in HIV-infected patients which requires T cell expansion leads to an 100-fold underestimate of CTL effector frequency. Since direct cytotoxicity measurements do not require cell growth, frequency measurements based on function would not be affected by antigen-induced apoptosis.

McMichael and co-workers used a similar assay as reported here to measure the frequencies of HIV gag-reactive T cells as calculated by PCR analysis of TCR chains of HIV-specific CTL clones. The frequency of HIV-reactive T cells using direct cytotoxicity assays was almost identical to that calculated by PCR, whereas the frequency as measured by LDA underestimated the frequency of HIV-reactive T cells (27). Moreover, the high frequency of HIV-reactive T cells as measured by PCR was confirmed using multimeric pep-

tide–MHC complexes that bound antigen-specific T cells (28). Specifically, MHC class I–A2 tetramers with HIV gag or pol peptide were used to identify HIV specific CD8⁺ in seropositive donors. Flow cytometric analysis revealed a high frequency of antigen specific CD8⁺ cells (0.77%) that supported frequency estimation based on the PCR method. Moreover, the high frequencies of MBPp85-99–reactive T cells in the subjects with MS are similar to the frequencies of cytochrome C reactive T cells calculated in mice using direct PCR measurement after immunization with antigen (29, 30). Thus, the frequency of circulating MBP-reactive T cells in active MS patients appears to be on the same order of magnitude as that observed with both MHC class I– and II–restricted recall antigens.

The high frequency of MBP-reactive T cells may reflect chronic stimulation of MBP-reactive T cells in the CNS. It is also possible that repeated challenges by cross-reactive microbes may induce selective T cell activation over time. The MBP reactive T cell clones expressing different TCR-α chains had similar dose response curves to MBPp85-99, yet exhibited markedly different fine specificities for peptides with different TCR contact residues. Moreover, these MBPp85-99–reactive T cell clones have been shown to recognize different cross-reactive viruses (31). Since only one of the MBPp85-99–reactive T cell populations was activated on a second time point tested, as measured by IL-2Rα chain expression, these data suggest that at this time point, the MBPp85-99–reactive T cells expressing the Hy-TDT CDR3 sequence were activated by a cross-reactive antigen and not the native MBPp85-99 sequence. Fluctuation of the MBPp85-99–specific clone with a CDR3-TDA sequence among IL-2Rα–positive and –negative populations over a time of 3 mo could also support such a possibility. Use of this approach to examine other subjects over longer periods of time may allow the determination of events that lead to the activation of autoreactive T cells in humans.

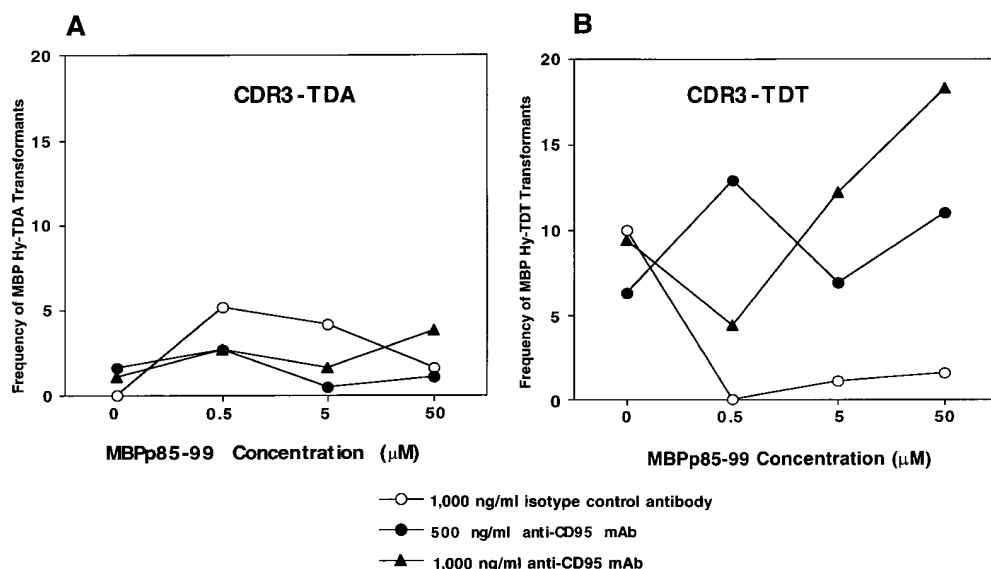Culture of peripheral blood T cells with MBPp85-99 ap-

Figure 3. The percentage of Vα3.1 transformants hybridizing to Hy-TDA and Hy-TDT probes was determined on day 0 (prestimulation) and after 72 h of culture with 0, 0.5, 5, or 50 μM MBPp85-99 either with control antibody alone (1,000 ng/ml isotype control antibody), or with 500 ng/ml or 1,000 ng/ml of anti-CD95 mAb (clone ZB4; Immunotech). (*A*) Frequency of Hy-TDA–positive transformants and (*B*) Hy-TDT–positive transformants. A total of 1,660 transformants were analyzed. The day 0 prestimulation values were 1.1% for Hy-TDA transformants and 3.4% for Hy-TDT transformants.

peared to induce Fas-mediated apoptosis of activated T cells. In this regard, there was a modest, approximately threefold, increase in the frequency of MBPp85-99–reactive T cells as measured by LDA in preliminary experiments when cultured in the presence of anti-CD95 mAb. While this may partly explain the low frequency of antigen-reactive T cells as measured by LDA, clearly other factors may also play a role. For example, it is possible that subpopulations of MBPp85-99–reactive T cells may represent regulatory T cells which are difficult to grow (32). Changes in culture conditions with the addition of other growth factors may also allow the expansion and measurement of greater numbers of circulating autoreactive T cells.

In interpreting these data, it is important to point out the limitations of extrapolating these data to all patients with MS. Sophisticated immunologic experiments in humans are greatly hampered by the outbred genotype of subjects. Thus, specific primers and probes for TCRs must be generated for each subject. Secondly, the patients with MS analyzed in these experiments were selected for further investigation because of previously demonstrated clonal expansion and clonal persistence of MBP-reactive T cells, and we do not believe that these data can be extrapolated to all subjects with the disease. The two normal subjects also had demonstrated the highest degree of clonal expansion observed in any of our control subjects, albeit not to the same degree as our subjects with MS (13). In fact, it is possible that MS is a heterogeneous disease where different myelin antigens are of importance in each individual. Nevertheless, these analyses of MBP-reactive T cells provide the first direct evidence for clonal expansion of MBP-reactive T cells in patients with MS and demonstrate that direct amplification of TCR chains can be used to quantitate circulating autoreactive T cells. Moreover, these data demonstrate that at least a subpopulation of patients with MS can have a very high frequency of activated autoreactive T cells which undergo Fas-mediated apoptosis upon antigen stimulation.

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