Clonal Expansion and Somatic Hypermutation of V_H Genes of B Cells from Cerebrospinal Fluid in Multiple Sclerosis

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Abstract

The cerebrospinal fluid (CSF) of multiple sclerosis (MS) patients is characterized by increased concentrations of immunoglobulin (Ig), which on electrophoretic analysis shows restricted heterogeneity (oligoclonal bands). CSF Ig is composed of both serum and intrathecally produced components. To examine the properties of intrathecal antibody-producing B cells, we analyzed Ig heavy-chain variable (V_H) region genes of B cells recovered from the CSF of 12 MS patients and 15 patients with other neurological diseases (OND). Using a PCR technique, we could detect rearrangements of Ig V_H genes in all samples. Sequence analysis of complementaritydetermining region 3 (CDR3) of rearranged VDJ genes revealed expansion of a dominant clone or clones in 10 of the 12 MS patients. B cell clonal expansion was identified in 3 of 15 OND. The nucleotide sequences of V_H genes from clonally expanded CSF B cells in MS patients demonstrated the preferential usage of the V_H IV family. There were numerous somatic mutations, mainly in the CDRs, with a high replacement-to-silent ratio; the mutations were distributed in a way suggesting that these B cells had been positively selected through their antigen receptor. Our results demonstrate that in MS CSF, there is a high frequency of clonally expanded B cells that have properties of postgerminal center memory or antibody-forming lymphocytes. (J. Clin. Invest. 1998. 102:1045-1050.) Key words: oligoclonal bands • intrathecal antibody-forming B cells • immunoglobulin heavychain variable gene • complementarity-determining region • autoimmunity

Introduction

Multiple sclerosis (MS)¹ is an inflammatory disease of the CNS with multifocal areas of demyelination (1, 2). Macrophages, T cells, and B cells are all present within the inflammatory le-

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sions (3–6). Although, as in the animal model experimental autoimmune encephalomyelitis, autoreactive T cells are considered to initiate the disease process (7, 8), antibodies are shown to contribute to the overall extent of tissue injury (9–11). In addition, memory B cells can contribute to antigen presentation, leading to activation of T cells (12, 13).

Intrathecal production of Ig can be documented in $\sim 90\%$ of MS patients, as calculated by formulae dependent on relative concentrations of serum and CSF Ig (14). The CSF Ig shows restricted heterogeneity as determined by electrophoresis (oligoclonal bands). Studies using the fluid component of MS CSF to examine the clonality and antigen specificity of Ig face the complication that this Ig is composed of both serum and intrathecal-derived components. Myelin protein-reactive antibodies are reported in the CSF of MS (15–17); oligoclonal bands, however, cannot be absorbed using myelin proteins (18). Although the limited numbers of cells present in the CSF of most MS patients precludes use of most conventional in vitro analyses of B cell properties, immunospot assays confirm the presence of myelin antibody-producing B cells in the CSF (19).

In this study, we analyzed the clonality of B cells present in the CSF of MS patients using a PCR-based approach that permitted characterization of rearranged Ig $V_{\rm H}$ genes expressed by these cells. Our results indicate that there is a high frequency of intrathecal B cell clonal expansion in MS CSF. Somatic hypermutations in these $V_{\rm H}$ genes were distributed in a way suggesting that these B cells had been positively selected through their antigen receptor.

Methods

Patients. CSF cell samples were obtained from each of 12 MS patients and 15 patients with other neurological diseases (OND). The MS patients included 11 females and 1 male with clinically or laboratory-supported definite MS diagnosis (20), with a mean age of 34±11 yr (Table I). The average duration of disease was 2±0.4 yr. The mean expanded disability status score (EDSS) was 3.2±1.3. All MS patients were categorized as having relapsing–remitting disease. Control patients were divided into inflammatory (I) and noninflammatory (NI) ONDs (Table I). Among the former were two patients with subacute illnesses diagnosed as acute disseminated encephalomyelitis and 1 with a remote history of Herpes Zoster encephalitis. Oligoclonal bands of all CSF samples were analyzed for presence of oligoclonal Ig bands by isoelectric focusing or agarose electrophoresis (Table I).

PCR amplification of CSF B cells. Total RNA was extracted from 2–8 ml CSF cells using an RNeasy kit (QIAGEN Inc., Chatsworth, CA). First-strand cDNA was synthesized using oligo d(T) as primer and avian myeloblastosis virus reverse transcriptase. V_H and VDJ genes were amplified via PCR (21) in a final volume of 50 μl reaction buffer (50 mM Tris-HCl, pH 9.0 at 25°C; 20 mM (NH₄)₂SO₄; 3.0 mM MgCl₂) containing 2 U of recombinant Taq polymerase, and 50 pmol primers (22, 23). PCR was carried out for 40 cycles under standard conditions (denaturation, 1 min at 94°C; annealing, 2 min at 52–56°C, extension 1 min at 72°C). Aliquots of the PCR product were analyzed

^{1.} Abbreviations used in this paper: CDR, complementarity-determining region; FR, framework region; MS, multiple sclerosis; OND, other neurological diseases; R:S, replacement-to-silence ratio.

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by electrophoresis in a 2% agarose gel (Sigma Chemical Co., St. Louis, MO) containing ethidium bromide. When PCR products showed negative results, nested PCR was performed.

Sequencing Ig $V_{\rm H}$ genes. PCR products were digested with EcoRI and BamHI before ligation into linearized M13 mp18, which was used to transfect *Escherichia coli* strain DH5 α according to the method of Hanahan (24). 8–12 white colonies were picked at random and grown overnight in 3 ml of Luria-Bertani (LB) medium. The double-stranded DNA template from the colonies containing $V_{\rm H}$ gene inserts was sequenced by the method of Sanger et al. (25).

Assignment of mutations. Mutations identified by comparing each sequence with germline sequences (University of Wisconsin Group, Madison, WI), were defined on the basis of nucleotide changes in the $V_{\rm H}$ segment, with any variability at the joining sites of the $V_{\rm H}$, D, and $J_{\rm H}$

Table I. Summary of Clinical and Laboratory Features of Patients Included in the CSF Analysis

		Cl	linical			CSF
(A) R-R MS		Age of	Disease	-		CSF
Samples	Sex	Onset	duration (yr)	MRI	ОВ	Cell number
1	F	48	3	+‡	_	9×10^4
2	F	31	4	+	+	8×10^4
3	F	24	1	+	+	4×10^4
4	F	48	2	+	+	3×10^4
5	F	22	6	+	+	8×10^4
6	F	28	1	1 SL	_	1.6×10^{5}
7	F	18	1	+	+	4×10^4
8	F	29	1	1 SL	+	1×10^{5}
9	F	36	1	+	+	6×10^4
10	F	21	1	+	+	2×10^4
11	M	41	4	+	+	1.6×10^{5}
12	F	43	1	NL	_	2.5×10^5

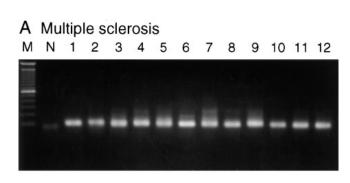
			Clinical	CSF				
(B) OND Samples	Sex Age		Diagnosis	ОВ	Cell number*			
I-OND								
1	F	64	ADEM	_	2×10^4			
2	F 24 ADEM		ADEM	_	$5 imes 10^4$			
3	F	86 Remote HZEM		_	UN			
NI-OND								
1	F	68	Headache	_	3×10^4			
2	F	66	Spinal cord	_	2×10^4			
Infarct								
3	M	47	Pseudotumor	_	5×10^4			
4	F	68	Neuropathy	_	6×10^4			
5	M	34	Migraine	_	3×10^4			
6	M	67	ALS	_	UN			
7	M	32	ALS	_	5×10^{3}			
8	F	40	Pseudotumor	_	2.5×10^{4}			
9	M	60	Hydrocephalus	_	4×10^4			
10	M	36	Neuropathy	+	2×10^4			
11	M	44	Spinocerebellar	_	1×10^{5}			
			degeneration					
12	M	57	Neuropathy	_	2×10^4			

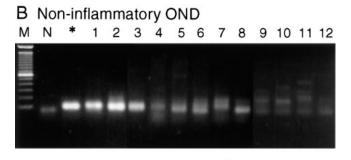
^{*}Cell number: the total numbers of white blood cells in the specimen (2–8 ml); †+: multiple lesions. ADEM, acute disseminated encephalomyelitis; ALS, amyotrophic lateral sclerosis; HZEM, Herpes Zoster encephalitis; NL, normal; OB, oligoclonal bands; SL, spinal lesion; UN, unknown.

gene segments not being classed as mutations, since they might result either from the insertion of N regions or from mutation. Two nucleotide exchanges in a single codon were scored as one replacement mutation.

Results

Immunoglobulin V_H rearrangements. To detect Ig V_H gene rearrangements of the CSF B cells, total RNA isolated from CSF cells from 12 patients with MS and 15 OND controls subgrouped into inflammatory (n=3) and noninflammatory (n=12) (Table I), was used to synthesize complementary (c) DNA. These cDNAs were then subjected to enzymatic gene amplification by the PCR technique. The results of such an amplification using primers for the VDJ region are shown in Fig. 1. PCR products from all MS and I-OND patients as well as three NI-OND controls showed high-density bands, suggesting a possible limited clonality pattern. PCR products from nine NI-OND cases (cases 4–12), showed only a faint smear, reflecting size differences in the V_H , D_H , and J_H regions in a polyclonal population of B cells (Fig. 1).





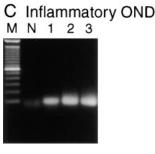


Figure 1. Ethidium–bromide-stained 2% agarose gel of FR3 PCR products from CSF B cells of the patients with MS or OND. (A) 12 MS samples; (B) 12 NI-OND samples plus a CNS paraffin tissue sample from a case of HIV⁺ non-Hodgkin's B cell lymphoma (*); (C) 3 I-OND samples. Lane M, 123 mol wt markers (size in basepairs); lane N, negative control.

Clonality of CSF B cells from patients with MS and OND, which showed a possible or probable clonal band of PCR products on ethidium-bromide-stained agarose gels, was determined by sequencing the expressed complementarity-determining region 3 (CDR3) genes. CSF B cells from 10 of 12 MS patients had dominant clonal or dual clonal rearrangements of CDR3. Clones derived from a given individual were identical in the $V_{\scriptscriptstyle H}$ -N-D-N-J $_{\scriptscriptstyle H}$ regions both in sequence and nucleotide length (Fig. 2). 3 of these 10 MS patients did not have oligoclonal bands detected by CSF electrophoresis. Conversely, both MS patients with polyclonal B cell sequences did have CSF oligoclonal bands. 3 of 15 controls were found to have dominant B cell clones, none of these patients had CSF oligoclonal bands. The sequences of the expressed D segments were compared with those of published germline D and DIR segments (26, 27). The combination of V_H, D_H, and J_H segments of these clonally expanded CSF B cells showed D-D fusion, D inversion, gene replacement and the addition of nucleotide sequences, termed N regions (Fig. 2).

Somatic hypermutations. The high frequency of dominant clonal expansion of CSF B cells expressed by MS patients raises the questions concerning the origin and characteristics of these cells. To elucidate these issues, the sequences of V_{H} genes of CSF B cells derived from four MS patients were analyzed. Fig. 3 shows the DNA and the deduced amino acid sequences of the V_H segments of the CSF B cells. The Ig V_H gene rearrangement in cases 1, 2, 3, and 10 used gene segments of V71-2, 4d154, 4d68, and 4d76 (28), which belong to the V_H IV families (Table II). The differences in nucleotide and predicted amino acid sequences when compared with the closest known germline V_H genes are summarized in Table III. Gene rearrangement mutations in the framework region (FR) of functional V regions are usually counterselected to preserve the structure of the antibody V domain. This results in replacement-to-silence (R:S) ratios below those expected on the basis of random mutagenesis. The combined R:S ratios for the FR

and CDR domains, derived from the sum of all mutated codons in the $V_{\rm H}$ sequences of the CSFB cells assignable to the germline gene segment, are shown in Table III. For cases 1, 2, 3, and 10 taken together, the average R:S ratio in the framework regions was 0.9 (expected \sim 2.9). The average R:S ratio in CDR1 and CDR2 was 4.0.

Discussion

Progress in identifying the characteristics of intrathecal B cells from MS has been hampered by the lack of experimental systems for analyzing the limited cell numbers derived from the CSF. In this study, we analyzed rearranged Ig V_H genes expressed by CSF B cells using PCR and sequencing techniques. The method used in this study was designed to amplify all V_H gene segments. We used a paraffin-embedded specimen of HIV-related primary CNS non-Hodgkin's B cell lymphoma (Fig. 2 B) and PBL samples from MS patients (data not shown) to confirm that we could detect dominant clonal sequences or polyclonal sequences, respectively. The sequences of CDR3 gene fragment of CSF B cells were analyzed for determining the clonality of these B cells. The criteria for establishing clonal relatedness were identity at the somatically formed V_H – D_H , D_H – J_H genes and use of the same sets of V_H D_H J_H genes. A dominant mono- or dual-clonal intrathecal B cell expansion was shown for 10 of 12 MS patients. When comparing nucleotide sequences of dominant CSF B cell clones from these patients, we found that each MS patient possessed individual patterns of V_H, D_H, and J_H combination. No apparent preferential utilization of any particular D_H gene segment set

Among the MS cases analyzed, cases with dominant monoclonal B cell sequences were found with and without CSF oligoclonal bands. The two MS cases with polyclonal sequences and positive oligoclonal bands further indicate that analysis of CSF oligoclonal band does not define the extent of B cell

Case	Identical	v	N	D	N	J H	
	Colonies	2					
MS		Sec. (4) State State Co.					
1.	6/7	TGTGCGAGC	TCA	(DM1) GGTGGATCCGG (A1) TGAATACGGAAA	A	TTTGACTA-CTGGGGCC	(JH4
2.	7/8	TGTGCGAGA	AC	(DN1) GGGTAG (LR4) AGGGAGGTGT		CTTCGGTATGGACGT-CTGGGGCC	(JH6
3.	7/7	TGTGCGAGAGA	TTTC	(LR3) AGGTCATACTG (XP3) TAGTGGTGATG	CCTG	CTACTACTACGGTATGGACGT-CTGGGGCC	(JH6
4.	4/7 3/7	TGTGCGAGA TGTGCCAGG	GGC	(DN1) AGGCGCAGCT (A1) CGAAGT GGATGGCTGGTACCCCA	CT TG	CTTGATTA-CTGGGGCC ATACTTTTGATGT-CTGGGGCC	(JH4 (JH3
6.	6/7	TGTGCGAAA		(DN1) GATATGGG (XP'1) CTATCACTATGCCTCGGGGAGTT	CC	TACTTTGACTC-CTGGGGCC	(JH4
8.	6/7	TGTGCGAGA	TATC	(XP'1) TTGGGGGATAAC (LR2) AACTCCA	AT	TTTGACTC-CTGGGGCC	(JH4
9.	4/7	TGTTCCACA		(LR1) GATGTACG	GA	ACCCG-CTGGGGCC	(JH5
10	4/6	TTCTAGAGA		(XP3) TCACTATGATAGTAGTGGTTATTG	CCA	ATGAATA-CTGGGGCC	(JH4
	2/6	TGCCACAGA	GGCGGG	(XP3)-TC	AGTCCA	CTTGACTA-CTGGGGCC	(JH4
11.	6/6	TGTGCGAGA	GTCGCCC	(XP'1) TTGCTATGGTTCGGGGAGT	ccc	TATGGACGT-CTGGGGCC	(JH6
12.	7/8	TGTGCGAGA	CTGG	(XP'1) GGTTCGGGGAGT	CGTCA	GACTA-CTGGGGCC	(JH4
OND-I							
1.	6/6	TGTGCGAGA	CTGG	(XP'1) GGTTCGGGGAGT	CGTCA	GACTA-CTGGGGCC	(JH4
OND-NI							
1.	6/6	TGTGCGAGA		(LR4) GGCCCCA (LR2) TTGTAGT	TATCCCA	AACTCTGCAATT -CTGGGGCC	(?)
2.	7/7	TGTGCGAGA	CAGTTC	(XP'1) GATCGGGGAG (DA5) AGCCAACT	A	CTTACTTTGACTA-CTGGGGCC	(JH4)
NHL-CN	s 5/7	TGTGCGAGA	GTCGGGCCCTT	(XP4) GTATTACGATTTTTGG	CCCTAC	TACATGGACGT-CTGGGGCC	(JH6)

Figure 2. The dominant CDR3 sequences of CSF B cells. Dominant sequences from each patient are grouped and subdivided into V_H , N, D, N, and J_H regions. Data from a case of primary CNS non-Hodgkin's Lymphoma (NHL) are also given. Names of the germline D and J_H genes with maximum homology to the segments used in the VDJ joining are shown in parentheses in the appropriate rows.

DNA se		ce																							
V71-2		GTG	CAG	CTG	CAG	CAG	TCG	GGC	CCA	GGA	CTG	GTG	AAG	CCT	TCG	GAG	ACC	CTG	TCC	CTC	ACC		R1	GT/C	an Cate
LP								CDR															R2		
V71-2 LP1									-A-													ATT			
V71-2 LP1							GG-	TAC C FR3	AAC																
V71-2 LP1 Case 2							AGC		GTG																
4d154 LM		CTG	CAG					GGC													ACC		R1 ACT	GTC	TCT
4d154 LM									TAC													CTG		TGG	ATT
4d154 LM	GGG t	AGT -A-	ATC	TAT A	TAT	AGT -T-	GGG	A	ACC	TAC C	TAC	AAC	CCG	TCC	CTC	AAG	AGT	CGA	GTC	ACC	ATA	TCC	GTA	GAC	ACG
4d154 LM Case 3								AAG	CTG																
4d68 ML								GGC														TGC			
4d68 ML								AAC	TGG											GGG	CTG		TGG	ATT	GGG
4d68 ML								ACC	AAC																
4d68 ML Case 1								FR3 CTG	AGC																
4d76 RA								GGC														TGC			
4d76 RA								TAC	TGG													TGG			
4d76 RA							T	AAC G	TAC																
4d76	AAC	CAG	TTC	TCC	CTG	AAG		FR3		GTG	ACC	GCC	GCG	GAC	ACG	GCT	GTG	TAT	TAC	TGT	GCG	n.ca			
RA		a																							
	aci																								
RA Aminal	aci	de s	eque:	nce.	Gln	-G-	T	Gly	Pro	Gly	Leu	 Val	Lys	Pro	Ser	Glu	Thr	Leu	Ser	Leu	Thr	F Cys			
RA Aminal Case 1 v71-2	Gln	Val	Gln	Leu	Gln	-G- Glu Tyr	Ser	Gly CDR	Pro 1 Ser Asn	Gly	Leu	Val	Lys	Pro 	Ser 	Glu 	Thr	Leu	Ser	Leu	Thr	F Cys F	Thr R2 Gly	Tyr	Ile
RA Aminal Case 1 v71-2 LP1 v71-2	Gln Gly Tyr	Val	Gln Ser	Leu Ile	Gln Ser	-G- Glu Tyr His	Ser Tyr	Gly CDR Trp CDR Tyr His	Pro 1 Ser Asn 2 Asn	Gly Trp 	Leu Ile	Val Arg 	Lys Gln 	Pro Pro	Ser Pro 	Glu Gly 	Thr Lys	Leu Gly 	Ser Leu 	Leu Glu 	Thr Trp Asp	Cys F Ile	Thr R2 Gly	Tyr	Ile
RA Aminal Case 1 v71-2 LP1 v71-2 LP1 v71-2 LP1 v71-2 LP1 v71-2 LP1	Gln Gly Tyr Gln Gln	Val Gly Tyr Phe	Gln Ser Ser Ser	Leu Ile Gly Leu	Gln Ser Gly	-G- Glu Tyr His	Ser Tyr Tyr Gly	Gly CDR Trp CDR Tyr His	Pro 1 Ser Asn 2 Asn	Gly Trp Pro	Leu Ile Ser	Val Arg Leu	Lys Gln Lys Asp	Pro Pro Ser	Ser Pro Arg	Glu Gly Val	Thr Lys Thr	Leu Gly Ile 	Ser Leu	Leu Glu Val	Thr Trp Asp	Cys F Ile Thr	Thr R2 Gly	Tyr	Ile
RA Aminal Case 1 v71-2 LP1 v71-2 LP1 v71-2 LP1 v71-2 LP1 case 2 4d154	Gln Gly Gln Gln Gln Gln Gln Gln	Val Gly Tyr Phe Phe Leu	Gln Ser Ser Gln	Leu Ile Gly Leu Leu	Gln Ser Gly Lys	-G- Glu Tyr His Thr Leu	Ser Tyr Tyr Gly Ser	CDR Trp CDR Tyr His FR3 Ser	Pro Ser Asn 2 Asn Val	Gly Trp Pro Thr Thr	Leu Ile Ser Ala	Val Arg Leu Ala	Lys Gln Lys Lys Lys	Pro Pro Ser Thr	Ser Pro Arg Ala	Glu Gly Val Val	Thr Lys Thr Tyr	Leu Gly Ile Cys Leu	Ser Leu Ser Ala	Leu Glu Val Arg	Thr Trp Asp Asp	Cys FIle Thr Thr Cys	Thr R2 Gly Ser Pro	Tyr	Ile
RA Aminal Case 1 v71-2 LP1 v71-2 LP1 v71-2 LP1 v71-2 LP1 Case 2	Gln Gln Gln Gln Gln Gln Gln Gly	Val Gly Tyr Phe Leu	Gln Ser Ser Gln Ser	Leu Gly Leu Leu Ile	Gln Ser Gly Lys Gln Ser Ser Ser Gly	Glu Tyr His Thr Glu Ser	T Tyr Tyr Gly Ser Ser	Gly CDR Trp CDR Tyr His FR3 Ser CDR Gly CDR	Pro Ser Asn 2 Asn Val Pro 1 Tyr	Gly Trp Pro Thr Thr Gly	Leu Ser Ala Leu Trp	Val Arg Leu Ala Val	Lys Gln Lys Asp Lys	Pro Pro Ser Thr Ile	Ser Pro Arg Arg Ala Ara	Glu Gly Val Glu Glu	Thr Lys Thr Thr Tyr Pro	Leu Cly Leu Pro	Ser Leu Ser Ala Ser	Leu Val Arg Leu	Thr Trp Asp Asp	F Thr Thr F Cys	R1 Thr Pro	Tyr Lys Lys	Ile Asn Ser Ile
RA Aminal Case 1 v71-2 LP1 v71-2 LP1 v71-2 LP1 v71-2 LP1 case 2 4d154 LM 4d154	Gln Gly Gln Gly Gly	Val Gly Tyr Phe Cly Gly Ser	Gln Ser Ser Ser Ser	Leu Ile Gly Leu Leu Tyr	Gln Ser Gly Lys Gln Ser	Glu Tyr His Thr Leu Glu Ser Lys	Tyr Tyr Gly Ser Ser	Gly CDR Trp CDR Tyr His FR3 Ser CDR Ser CDR Ser	Pro 1 Ser Asn 2 Asn Val Pro 1 Tyr	Gly Trp Pro Thr Thr Gly Tyr	Leu Ile Ser Ala Leu Trp	Val Arg Leu Ala Val	Lys Gln Lys Lys Trp	Pro Pro Ser Thr Pro Ile	Ser Pro Arg Ala Ser Arg Leu	Glu Gly Val Val Glu	Thr Lys Thr Tyr Thr Ser	Leu Gly Cys Leu Arg	Ser Leu Ser Ala Ser Gly Val	Leu Glu Val Arg Leu Lys Gln	Thr Trp Asp Asp	F Cys Thr Thr Cys F Leu	Thr R2 Gly Ser Pro R1 Thr R2 Glu Val	Tyr Lys Val Trp Asp	Ile Asn Ser Ile
RA Aminal Case 1 v71-2 LP1 v71-2 LP1 v71-2 LP1 v71-2 LP1 case 2 4d154 LM 4d154 LM	Gly Gly Gly Ser	Val Gly Tyr Phe Cal Gly Ser Asn	Gln Ser Ser Gln Ser	Leu Gly Leu Tyr Asn	Gln Ser Gly Lys Gln Ser	Glu Tyr His Thr Leu Ser Lys Ser Ile Ser	Ser Tyr Gly Ser Ser	Gly CDR Trp CDR Try His FR3 Ser CDR Ser CDR Ser FR3 FR3	Pro Ser Asn 2 Asn Val Pro 1 Tyr Thr	Gly Trp Pro Thr Thr Thr Tyr His	Leu Ser Ala Trp Tyr Ser	Val Leu Ala Val Gly Val	Lys Gln Lys Lys Trp Pro Thr	Pro Pro Ser Ile Ser Ala	Ser	Glu Gly Val Glu Glu Lys Asp	Thr Lys Thr Thr Tyr Thr Thr	Leu Gly Ile Cys Leu Arg Ala	Ser Leu Ser Ala Ser Gly Val Val	Leu Glu Val Arg Leu Lys Gln Thr Tyr	Thr Trp Asp Asp Thr Gly Tle	FF Cys Thr Thr FF Cys Ser Cys	Thr R2 Gly Ser Pro R1 Thr Clu	Tyr Lys Val Trp Asp	Ile Asn Ile Thr
RA Aminal Case 1 v71-2 LP1 v71-2 LP1 v71-2 LP1 v71-2 LP1 d154 LM 4d154 LM 4d154 LM 4d154 LM Case 3	Gly	Val Gly Tyr Phe Gly Ser Asn Lys	Gln Ser Ser Ser Ile Gln Gln Gln Gln Gln	Leu Gly Leu Tyr Asn Gln Leu	Gln Ser Gly Lys Gln Tyr Phe	Glu Glu Ser Lys Ser Ile Ser Gly	Ser Tyr Gly Ser Ser Gly Ser	Gly CDR Trp CDR Tyr His FR3 Ser CDR Ser Arg FR3 Lys Gly	Pro 1 Ser Asn 2 Asn Val Pro 1 Tyr Lue	Gly Trp Pro Thr Thr Tyr His Ser	Leu Tle Ala Trp Tyr Ser	Val Arg Leu Ala Gly Val Val	Lys Gln Lys Lys Trp Trp Pro Thr	Pro Ser Ile Ala	Ser Pro Arg Ala Arg Arg Arg Ser Arg Ser Ser Arg Arg Arg Arg Ala Arg Ser Ser	Glu Gly Val Glu China Glu China Glu China Glu China Glu China Gly China Chin China China China China China Chin China China Chin China Chin	Thr Lys Thr Tyr Thr Thr Thr	Leu Gly Cys Leu Arg Pro	Ser Leu Ser Ala Cly Val Val Ser Val	Leu Glu Val Leu Lys Gln Thr Tyr	Thr Trp Asp Asp Thr Thr Trr Trr Trr	F Cys Thr Thr Thr Cys Ser Cys	R1 Thr Pro R2 Gly Ser Pro R1 Thr Ala Ala	Tyr Lys Val Trp Asp Arg Asn	Ile Asn Ile Thr
RA Aminal Case 1 v71-2 LP1 v71-2 LP1 v71-2 LP1 v71-2 LP1 dd154 LM dd68	Gln	Val Gly Tyr Phe Gly Ser Asn Lys Glv	Gln Ser Ser Gln Ser Gln Ser Gln Ser	Leu Leu Tyr Asn Gln Leu	Gln Ser Gly Lys Gln Ser Tyr Phe	Glu Tyr His Thr Leu Ser Lys Ser Glu Ser Gly Ser	Ser Tyr Gly Ser Ser Leu Ser	Gly CDR FR3 Gly CDR Ser CDR Ser CDR Gly CDR Arg FR3 Arg Gly CDR Arg Arg Arg Arg Arg Arg Arg Arg CDR	Pro 1 Ser Asn 2 Asn Val Pro 1 Tyr Lue Pro 1 Trp	Gly Pro Thr Thr Thr Gly Tyr Gly Tyr Tyr Tin	Leu Ser Leu Trp Tyr Ser Leu Ser	Val Arg Leu Ala Gly Val Trp	Lys Gln Lys Lys Trp Pro Thr	Pro Pro Ser Thr Pro Ala Arq	Ser Arg Leu Ala	Glu Glu Glu Glu Glu Glu Gln Gln Gln Glr	Thr Lys Thr Tyr Thr Fro Thr	Leu Gly Ile Cys Leu Pro Arg Ala Gly	Ser Leu Ser Ser Val Ser Val Ser Lys	Leu Glu Val Arg Leu Lys Gln Thr Tyr Leu Gly	Thr Trp Asp Thr Gly Tyr Tyr Leu	FF Cys FIle Thr Thr Cys FC Cys FG Glu	R1 Thr	Tyr Lys Val Trp Asp Arg Asn Val	Ile Asn Ile Thr Ser Gly
RA Aminal Case 1 v71-2 LP1 v71-2 LP1 v71-2 LP1 v71-2 LP1 case 2 4d154 LM 4d154 LM 4d154 LM 4d154 LM Case 3	Gln Gly Gly Gly Gln Gly Gly Gln Gly Gly	Val Gly Phe Leu Gly Ser Asn Uys Tyr	Gln Ser Ser Gln Ser Gln Ser Tyr	Leu Leu Tyr Asn Gln Leu His	Gln Ser Gly Lys Gln Ser Phe Ser Ser	Glu Glu Ser Lys Ser Ile Gly Gly Gly Gly	Ser Ser Leu Ser	Gly CDR Trp CDR Tyr His Ser CDR Ser CDR Arg Arg Gly CDR CDR Trn	Pro 1 Ser Asn 2 Asn Val Pro 1 Tyr Lue Pro 2 Asn	Gly Pro Thr Thr Thr Gly Tyr Tyr Tipr Tyr Tyr Tyr Tyr Tyr	Leu Trp Tyr Ser Leu Leu Asn	Val Arg Leu Ala Gly Val Trp Pro	Lys Gln Lys Lys Trp Thr Lys Val	Pro Pro Ser Thr Pro Ala Pro Arg	Ser Arg Ala Ala Ser Ala Leu His	Glu Glu Glu Glu Glu Glu Glu Gln Lys Glu Fro	Thr Lys Thr Tyr Thr Pro Thr Thr Arg	Leu Gly Tle Cys Leu Pro Arg Ala Pro Gly Val	Ser Leu Ser Ser Val Ser Val Lys	Leu Glu Val Arg Leu Lys Gln Thr Tyr Gly Leu Lieu Lieu Lys Gln	Thr Trp Asp Asp Thr Gly Tyr Tyr Thr Ser	FF Cys Thr Thr Thr Cys F Cys Cys Cys Cys Val	Thr R2 Gly Ser Pro R1 Thr R2 Glu Val Ala R1 Ala R1 Asp	Tyr Lys Val Trp Asp Arg Arg Trb Trb Trb Trb	Ile Asn Ile Thr Gly Ser
RA Aminal Case 1 v71-2 LP1 v71-2 LP1 v71-2 LP1 v71-2 LP1 dd154 LM dd68 ML	Gln Gly Gly Gln Gly Gly Gln Gly Gly Lys	Val Gly Phe Gly Ser Asn Ual Gly Tyr Phe Tile	Gln Ser Ser Gln Ser Gln Ser Tyr Gly	Leu Leu Tyr Asn Gln Leu His	Gln Ser Gly Lys Gln Ser Gln Ser Gln Ser Gln Ser Gln Ser Ser Gln Ser	Glu Tyr His Thr Leu Ser Lys Ser Ile Ser Gly Gly Leu Leu Lys	Ser Tyr Gly Ser Ser Ser Leu Ser Ser Leu Lys	Gly -CDR Tyr Hiss FR3 Ser -CDR Ser Arg Gly -CDR CDR CDR CDR CDR CDR CDR CDR CDR CDR	Pro 1 Ser Asn 2 Asn Val Tyr 2 Thr Lue Pro 1 Trp Ser Asn Ser Asn Ser Ser Ser Ser Ser Ser Ser Ser	Gly Trp Pro Thr Thr Gly Tyr His Ser Trp Trp Ser	Leu Ile Ser Ala Trp Tyr Leu Ser Asn Val	Val Arg Leu Ala Gly Val Trp Pro	Lys Gln Lys Lys Trp Thr Lys Val Ser	Pro Pro Ser Thr Ala Pro Arg	Ser Pro Arg Ala Ser Arg Leu Ala Ser Arg Ala	Glu Gly Val Glu Glu Glu Gln Gln Lys Gly Fro Ser	Thr Lys Thr Tyr Thr Pro Thr Arg	Leu Gly Cys Leu Pro Arg Ala Cys Val	Ser	Leu Glu Val Lys Gln Thr Tyr Leu Gly Tyr Tyr Tyr	Thr Trp Asp Thr Gly Ttyr Tyr Cys	FF Cys FG Cys FG Cys FG Cys Val	Thr R2 R2 Gly Ser Pro R1 Thr Ala Ala Ala Arg	Tyr Lys Val Trp Asp Arg Arg Trb Trb Trb Trb	Ile Asn Ile Thr Gly Ser
RA Aminal Case 1 v71-2 LP1 v71-2 LP1 v71-2 LP1 case 2 4d154 LM 4d154 LM dd154 LM dd154 LM dd154 LM dd68 ML dd68	Gln Gly Gly Gln Gly Gly Gln Gly Gln Gly Gln	Val Gly Phe Gly Ser Asn Val Gly Val Val	Gln Ser Ser Gln Ser Gln Ser Tyr Gly Asn	Leu Leu Tyr Asn Gln Leu His	Gln Ser Gly Lys Gln Ser Tyr Phe Ser Gly Ser Gly Ser Gly	Glu Tyr His Thr Leu Glu Ser Leu Gly Ser Gly Gly Gln Gln	Ser Tyr Gly Ser Ser Ser Tyr Tyr Tyr Tyr Ser Tyr Ser Tyr Tyr Tyr Tyr Tyr Tyr Tyr Tyr Tyr Ty	Gly CDR Gly CDR Gly CDR CDR Gly CDR CDR CDR CDR CDR CDR CDR CD	Pro 1 Ser Lys Asn Pro 2 Asn Tyr Thr Lue Pro 2 Asn Asn Arg Asn Ala	Gly Trp Pro Thr Thr Tyr His Ser Trp Tyr Gly	Leu Tle Ser Ala Leu Trp Tyr Ser Leu Leu Leu Leu Leu	Val Arg Leu Ala Val Gly Val Trp Pro	Lys Gln Lys Asp Trp Pro Thr Lys Val Lys Ser Ala	Pro Pro Ser Thr Pro Ala Leu Leu	Ser Pro Arg Ala Ser Arg Leu Ala Lys Ser Ser	Glu Gly Val Glu Glu Glu Glu Gln Cly Asp Pro Gly Fro Glu Gly	Thr Lys Thr Tyr Thr Pro Ser Thr Arg	Leu Gly Cys Cys Leu Arg Ala Cys Leu	Ser Leu Ser Ala Ser Gly Val Val Lys Thr Tyr	Leu	Thr Trp Asp Asp Thr Gly Thr Tyr Cys Thr	FF Cys For	Thr R2 Gly Ser Pro R1 Thr Ala Ala Ala Asp Arg	Tyr Lys Val Trp Asp Arg Asn Val Thr Arg	Ile Ser Thr Ser Tyr
RA Aminal Case 1 v71-2 LP1 v71-2 LP1 v71-2 LP1 v71-2 LP1 dd154 LM 4d154 LM 4d154 LM 4d154 LM 4d154 LM 4d68 ML 4d68	Gln Gly Gly Gln Gly Gln Gly Gln Gln Gly	Val Gly Tyr Phe Gly Ser Asn Usl Gly Val Gly Val Gly	Gln Ser Ser Gln Ser Gln Ser Gln Ser Gln Ser Gln Ser Gly Asn Gln Ser	Leu Leu Tle Tyr Asn Gln Leu Leu Leu Leu Phe	Gln Ser Gly Lys Gln Ser Tyr Phe Gln Ser Gly Ser Gly Ser Gly	Glu Tyr His Thr Leu Ser Lys Ser Gly Gly Gly Gly Gly	Ser Tyr Tyr Gly Ser Ser Ser Tyr Tyr Tyr Tyr Tyr Ser Tyr Tyr Tyr Tyr Tyr Tyr Tyr Tyr Tyr Ty	Gly CDR Ser CDR Ser CDR Ser CDR Ser CDR FR3 Lys CDR Thr CDR Thr Try This	Pro 1 Ser Asn 2 Asn Val Tryr Tryr Lue Pro 1 Trp Asn Arg Asn Arg Ala Trp	Gly Trp Pro Thr Thr Gly Tyr Tyr His Ser Gly Trp Gly	Leu Ser Ala Leu Trp Ser Asn Leu Leu Trp Tyr	Val Leu Ala Gly Val Trp Pro Thr	Lys Gln Lys Lys Trp Pro Thr Lys Val Lys Glu Arg	Pro Pro Ser Thr Ile Ala Pro Arg Gln	Ser Pro Arg Ala Ser Arg Leu Ala Lys Ser Asp Pro	Glu Gly Val Glu Glu Glu Glu Glu Glu Gln Cly Gly Pro Gly Pro Gly	Thr Thr Tyr Thr Pro Ser Thr Arg Ala	Leu Gly Cys Cys Pro Arg Arg Gly Val Leu Lys	Ser Leu Ser Gly Val Lys Thr Tyr Ser Gly Gly	Leu Val Arg Leu Lys Gln Thr Tyr Leu Gly Ile Leu Leu Leu Leu Leu Leu Leu	Thr Asp Asp Thr Gly Ttr Ttr Cys Thr Gen Cys	FF Cys FF Cys FF Cys FF Cys FF Cys Cys FF Cys FF Cys FF Cys FF Cys FF Typ FF Typ	Thr R2 Gly Ser Pro R1 Thr R2 R2 Gly Ala Ala Ala Ala Asp Asp Arg R1 R1 Ala Ala R1 Ala Ala R1 R1 Ala	Tyr Lys Val Trp Asp Arg Asn Val Thr Arg	Ile Ser Thr Ser Tyr
RA Aminal Case 1 v71-2 LP1 v71-2 LP1 v71-2 LP1 case 2 4d154 LM 4d154 LM dd154 LM 4d154 LM 4d68 ML 6d68 ML	Gln Gly	Val Gly Tyr Phe Gly Ser Asn Val Gly Control Co	Gln Ser Ser Gln	Leu Leu Leu Tyr Asn Gln Leu His Phe	Gln Ser Gly Ser Gln Ser Gln Ser Gly Gln Gln Gln Gln Gln Gln Gln Gly	Glu Tyr His Thr Leu Ser Lys Ser Ile Ser Gly Gly Gln Gln Gly Thr	Ser Ser Ser Leu Ser Thr Tryp	Gly CDR Gly CDR CDR Gly CDR CDR CDR CDR CDR CDR CDR CD	Pro 1 Ser Asn Val Pro 1 Tyr Thr Lue 1 Trp Asn Ala Ala Trp Tyr Tyr Tyr	Gly Pro Thr Thr Thr Gly Tyr His Ser Trp Gly Ser Asn	Leu Ser Ala Trp Tyr Ser Leu Asn Val Leu Pro	Val Arg Leu Ala Gly Val Trp Fro Thr Leu Ser	Lys Gln Lys Lys Trp Pro Thr Lys Val Lys Ser Ala Lys Lys	Pro Pro Ser Thr Ala Pro Arg Leu Leu Lys	Ser Pro Arg Ala Arg Leu Ala His Ser Pro Ser Ser	Glu Gly Val Glu Glu Glu Glu Glu Glu Glu Glu Glu Gl	Thr Lys Thr Tyr Thr Pro Thr Arg Ala Thr Gly	Leu Gly Cys Leu Arg Pro Ala Cly Leu Lys Thr	Ser Leu Ser Ala Gly Val Lys Thr Tyr Ser Gly	Leu Glu Val Leu Lys Gln Thr Leu Lys Lys Leu Leu Ser Ser	Thr Trp Asp —— Asp —— Thr Gly Tyr Thr Cys Cys Thr Cys	F F Cys Cys Cys Cys Glu Ala F Trp Asp	Thr — R2 Gly — Ser Pro R1 Thr — Pro R2 Glu — Ala — Ala — Ala — Asp — Arg — Arg — Arg — Thr — T	Tyr Lys Trp Asp Arg Asn Val Thr Arg Val Ser	Ser Ille Thr Ser Gly Tyr Phe Glu Lys

Figure 3. V_H gene segments and translated peptides. The nucleotide and deduced amino acid sequences of V_H germline gene segments are shown in comparison with germlines v71-2 (case 1), 4d154 (case 2), 4d68 (case 3), and 4d76 (case 10). Conventionally defined CDR are indicated. Dashes, identity to the reference sequence. Upper-case and lower-case letters, replacement and silent mutations, respectively.

Table II. Utilization of VH Gene Segments

Case	VH family	Closest germline	Identity %
1	VH IV	v71-2	94
2	VH IV	4d154	94
3	VH IV	4d68	94
10	VH IV	4d75	93

clonality. In this regard, none of the I-OND or NI-OND cases with dominant B cell sequences had detectable oligoclonal bands. The sequences of $V_{\rm H}$ genes derived from the four MS patients analyzed showed preferential utilization of the $V_{\rm H}$ IV family. Owens et al., analyzing Ig $V_{\rm H}$ genes from demyelinating lesions of a single case of acute MS, also found restricted usage of the $V_{\rm H}$ IV family (29). Analysis of Ig $V_{\rm H}$ genes of CSF B cells permits study of a wider array of patients than can be performed on autopsy tissue, and is particularly suited for patients with relatively early stages of disease.

Positive selection of clones bearing somatic mutations, resulting in an increase in affinity of the surface receptor for antigen, has been shown to be the primary mechanism underlying the process of affinity maturation during a specific immune response (30–33). Somatic hypermutation of Ig V region genes is generally believed to occur in the germinal centers (34–36). In this study, the role of positive selection in the dominant expansion of CSF B cells was investigated. Compared with the germline, the V_H genes of CSF B cells from four patients with MS contained at least 16 substitutions, distributed in a pattern characteristic of antigen-driven affinity maturation, i.e., the somatic mutations were highly concentrated in the CDR or FR, with a clustering of replacement (R) mutations in the CDR, but only a few in the FR. The R:S mutation ratios in the CDRs (4.0) and FRs (0.9) were comparable to those in the V genes of high-affinity murine antibodies and autoantibodies (30, 32, 35) and were significantly higher or lower respectively, than the theoretical R:S value of a protein \sim 2.9, calculated for somatic mutations occurring randomly in a gene encoding a protein whose structure need not be preserved. A higher CDR R:S mutation ratio reflects the positive selective pressure of an antigen on those gene products that come into close contact with antigen, while a lower FR R:S mutation ratio reflects the negative pressure for mutant selection applied to structural components that need to be conserved (37). This pattern is consistent with the notion of an antigen-driven selection of antibodies with high-affinity antigen-binding sites. An alternative explanation could be that these mutations might result from polymorphic variation of the Ig V_H gene. However, it seems unlikely.

Table III. Differences in Nucleotide Sequences of Dominant Clone from CSF Cells of R-R MS

	Nı	umber of n	nucleotid	Tot	al	R S			
Case	FR1	CDR1	FR2	CDR2	FR3	CDRs	FRs	CDRs	FRs
1	1	2	2	5	6	7	9	6.0	0.3
2	1	3	2	6	4	9	7	3.0	0.5
3	4	0	0	5	9	5	13	3.0	1.0
10	5	3	1	6	6	9	12	3.0	2.0

We have successfully amplified the entire gene segments of $V_{\rm H}$ gene family and germline from PBL of case 1. Comparison with the appropriate published germ fragment sequences shows 100% identity with the published V71-2 gene. The results demonstrate that, even when taking allelic polymorphism into account, the Ig $V_{\rm H}$ genes of CSF B cells from MS patients clearly show the somatic hypermutation. The presence of D–D recombination in CDR3 regions of CSF B cells provides further evidence for antigen-driven selection. This type of D–D recombination event has also been reported in response to hapten–antigen complex and the random terpolymer GAT in mice and represents an important event in determining idiotype expression and antigen-binding affinity (38).

Our data provide direct evidence that intrathecal clonally expanded B cells from the CSF of MS are T cell dependent hypermutated postgerminal center antibody-forming or memory lymphocytes that have undergone antigen selection. This finding might have been predicted from previous description of distinct oligoclonal T cell populations being present in the CSF of MS (39). The direct sequencing studies of MS lesions further indicated restricted T cell receptor V gene usage, with at least one sequence corresponding with that of a previously identified myelin basic protein reactive T cell clone (40). Further studies would need to address the nature of the antigen/s, which drive the B cell clonal expansion in MS.

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