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 complementarity-determining 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 heavy-chain 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 lesions (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_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_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 $(NH_4)_2SO_4$; 3.0 mM $MgCl_2$) 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

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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_H genes. PCR products were digested with EcoRI and BamHI before ligation into linearized M13 mp18, which was used to transfect *Escherichia coli* strain DH5α 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_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_H segment, with any variability at the joining sites of the V_H, D, and J_H 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.

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

	Clinical				CSF	
(*A*) R-R MS Samples	Sex	Age of Onset	Disease duration (yr)	MRI	OB	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		OB	Cell number*
I-OND						
1	F	64	ADEM		–	2×10^4
2	F	24	ADEM		–	5×10^4
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 degeneration		–	1×10^5
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.

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).

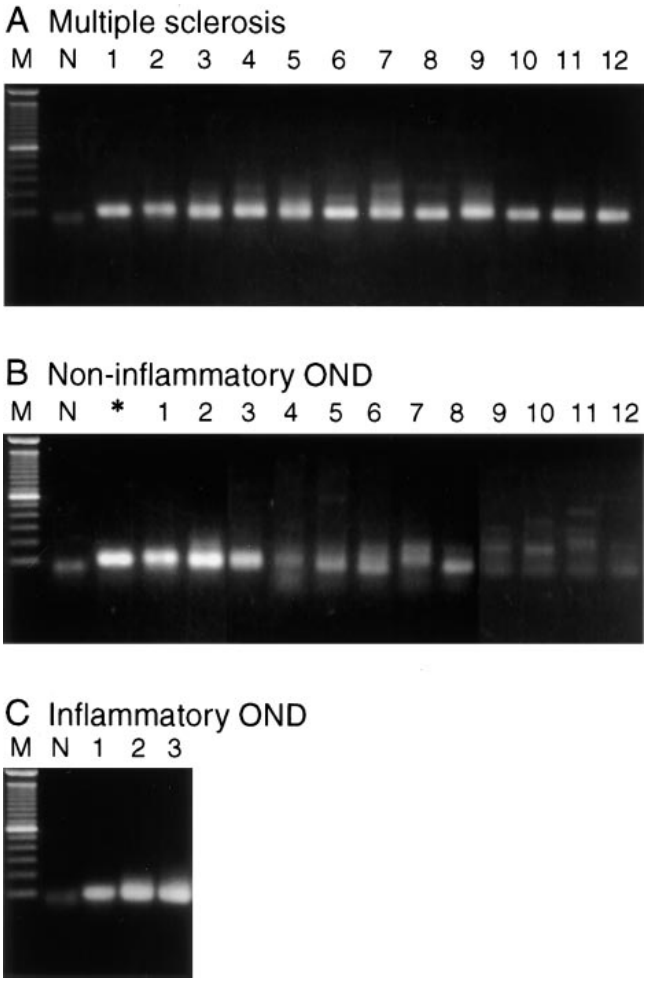

Figure 1. Ethidium–bromide-stained 2% agarose gel of FR3 PCR products from CSF B cells of the patients with MS or OND. (*A*) 12 MS samples; (*B*) 12 NI-OND samples plus a CNS paraffin tissue sample from a case of HIV^+ non-Hodgkin's B cell lymphoma (*); (*C*) 3 I-OND samples. Lane *M*, 123 mol wt markers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

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 Colonies	V	N	D	N	JH	
MS							
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	CTACTACTACTACGGTATGGACGT-CTGGGGCC	(JH6)
4.	4/7	TGTGCGAGA	GGC	(**DN1**)AGGCGCAGCT(**A1**)CGAAGT	CT	CTTGATTA-CTGGGGCC	(JH4)
	3/7	TGTGCCAGG		GGATGGCTGGTACCCCA	TG	ATACTTTTGATGT-CTGGGGCC	(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**)-T-----C----------	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	TATCCCAACTCTGCAATT	-CTGGGGCC	(?)
2.	7/7	TGTGCGAGA	CAGTTC	(**XP'1**)GATCGGGGAG(**DA5**)AGCCAACT	A	CTTACTTTGACTA-CTGGGGCC	(JH4)
NHL-CNS	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.

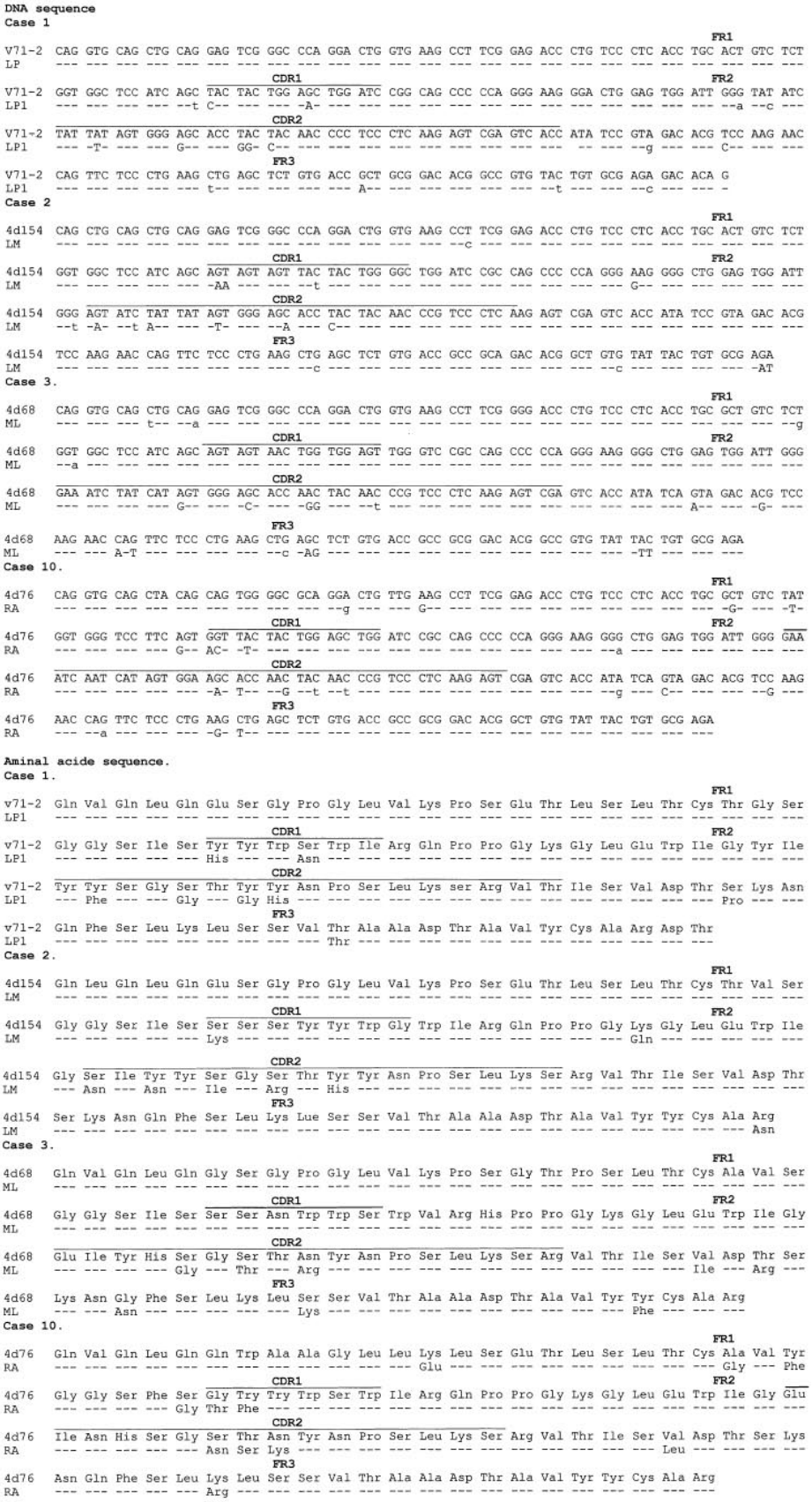

Figure 3. V_H gene segments and translated peptides. The nucleotide and deduced amino acid sequences of V_H germline gene segments are shown in comparison with germlines v71-2 (case 1), 4d154 (case 2), 4d68 (case 3), and 4d76 (case 10). Conventionally defined CDR are indicated. *Dashes*, identity to 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_H genes derived from the four MS patients analyzed showed preferential utilization of the V_H IV family. Owens et al., analyzing Ig V_H genes from demyelinating lesions of a single case of acute MS, also found restricted usage of the V_H IV family (29). Analysis of Ig V_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

	Number of nucleotide differences					Total		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_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_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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