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Accumulation of Clonally Related B Lymphocytes in the Cerebrospinal Fluid of Multiple Sclerosis Patients[1]

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The accumulation of B lymphocyte clones in the cerebrospinal fluid (CSF) of patients with multiple sclerosis (MS) and patients with other neurological disorders was investigated using PCR technologies. Oligoclonal B cell accumulations were detected in 10 of 10 MS patients, but only in 3 of 10 of the patients with other neurological disorders. Analyses of the Ig V(D)J sequences on the CSF from MS patients disclosed that V_H3 and V_H4 genes were extensively mutated compared with germline sequences. Moreover, a substantial proportion of the molecular clones analyzed shared the same third CDR of the H chain variable region gene (HCDR3) and the same V_H genes, albeit with different numbers and locations of point mutations, thus indicating an ongoing process of intraclonal diversification. A larger number of clonally related V_H sequences could be obtained by using a V_H3 gene-specific PCR so that genealogical trees depicting the process of diversification could be drawn. Analyses of the Ig V(D)J from the CSF of a patient with viral meningitis and oligoclonal B cell accumulations revealed that V_H3 genes were extensively mutated. However, no intraclonal diversification could be observed even using V_H3 gene-specific PCR methodologies. Clone-specific PCR and sequencing was used to detect the V(D)J found in the CSF of one MS patient in the PBL of the same patient. Only 1/3 of the V(D)J sequences investigated could be demonstrated in the PBL, indicating that the V(D)J genes utilized by B cells in the CSF are much less represented in the PBL. Collectively, the data suggest that in MS there is a compartmentalized clonal expansion. ***The Journal of Immunology,*** **2000, 164: 2782–2789.**

Multiple sclerosis (MS)[3] is a chronic demyelinating disease of the CNS for which two alternative etiological explanations are traditionally offered (1, 2). One postulates that an infection by a virus or by another pathogen results in the recruitment of inflammatory cells within the CNS; such inflammatory reactions may eventually contribute to the onset of autoimmunity. The other proposes that the disease is initiated by an autoimmune reaction primarily directed toward myelin Ags.

The inflammatory infiltrates of MS are comprised of T cells, macrophages, and B cells. It is generally assumed that T cells play a pivotal role in initiating the inflammatory lesions, as indicated by studies on experimental animal models, especially experimental autoimmune encephalomyelitis (3–5). However, the production of autoantibodies, particularly those reactive with myelin, has relevance since they can contribute to the process of demyelinization (6–9). The involvement of B cells in MS is suggested by a number of observations. For example, the cerebrospinal fluid (CSF) of MS patients is characterized by the presence of Ig molecules with restricted isoelectric focusing (IEF) mobility (10). These bands are not usually detected in the plasma and there is evidence indicating that they are produced intrathecally (11–13). Moreover, micromethods indicate that B cells producing anti-myelin Abs exist in the CSF of MS patients (14–16). However, the number of B cells present in the CSF is too low to permit studies with the classical methods of cellular immunology (17, 18).

The advent of PCR methodologies and the recent understanding of the control of Ig V_H and V_L gene assembly in B cells have made it possible to collect information on the developmental and maturational history of B cells by studying their Ig V region genes. During a T cell-dependent response, B cells accumulate point mutations in their V_H and V_L genes and B cells expressing those V gene variants that lead to increased affinity for the stimulating Ag are selected for survival and clonal expansion (19). This selection takes place mainly, but not necessarily (20–22), in the germinal centers of the lymphoid organs (19). Moreover, among these stimulated and Ag-selected B cells, there may be a predominance of B cells that are the progeny of a single precursor and share the same rearranged V_H or V_L gene, albeit with different numbers and distributions of point mutations. Thus, the accumulation of point mutations in clonally related V gene sequences within a given B cell population can be used as a marker of an ongoing response to stimulating Ag(s). By using PCR methodology and the above-illustrated criteria, we have collected evidence for an ongoing B cell response in the CSF of MS.

Materials and Methods

Patients

CSF and PBMC samples were obtained from each of 10 MS patients and 10 patients with other neurological disorders (OND). The MS patients, with clinically or laboratory-supported definite MS diagnosis, were

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[3] Abbreviations used in this paper: MS, multiple sclerosis; CSF, cerebrospinal fluid; IEF, isoelectric focusing; CDR, complementarity-determining region; HCDR3, third CDR of the heavy chain variable region gene; FR, framework region; OND, other neurological disorders; RR, relapsing-remitting; SP, secondary progressive; R, replacement mutation; S, silent mutation.

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Table I. *Major features of the MS patients*

MS Patients	Clinical				CSF Cells/mm^3	CSF Cell No. PCR[a]	Oligoclonal IgG Bands (IEF)[b]		HCDR3 Band Analysis (PCR)[c]	
	Sex	Age (yr)	Duration	Course			CSF	S	CSF	PB
1A	M	17	1 year	RR	98	125×10^3	+	−	Oligo	Poly
2A	M	59	8 years	SP	15	150×10^3	+	−	Oligo	Poly
3A	F	57	9 years	SP	14	81×10^3	+	−	Oligo	Poly
4A	F	33	1 mo	RR	9	90×10^3	+	−	Oligo	Poly
5A	M	31	6 years	RR	11	75×10^3	+	−	Oligo	Poly
6A	F	37	5 years	RR	28	110×10^3	+	−	Oligo	Poly
7A	M	23	1 mo	RR	15	150×10^3	+	−	Oligo	Poly
8A	F	23	7 years	RR	14	112×10^3	+	−	Oligo	Poly
9A	M	18	1 mo	RR	14	190×10^3	+	−	Oligo	Poly
10A	F	24	2 years	RR	25	225×10^3	+	−	Oligo	Poly

[a] The total number of cells used for the PCR.
[b] Presence (+) or absence (−) of distinct bands.
[c] Oligo refers to the presence of discrete bands observed in at least two major V_H families (V_H3/V_H4); poly refers to the presence of small, smearing bands.

categorized according to clinical course as having either relapsing-remitting (RR, patient 1A and patients 4A-10A, see Table I) or secondary progressive disease (SP, patients 2A and 3A, Table I). All cases were free of immunosuppressive treatment and had not received steroid therapy in the 6 mo preceding lumbar puncture. CSF examination was conducted for diagnostic purposes or during exacerbation of neurological symptoms, and each patient gave informed consent to perform the procedure. The OND patients included a variety of nondemyelineating disorders as indicated in Table II.

PCR methodologies

Total RNA was extracted from either CSF cells (range, 1.3×10^4–2.5×10^5 cells, see Tables I and II) or PBMC (2.5×10^4– $\times 10^6$) using RNA-Clean System (TB Molbiol, Berlin, Germany) and was reverse transcribed for first cDNA synthesis as detailed (23).

Genomic DNA was purified from either CSF cells or PBMC by cell lysis followed by digestion with proteinase K, "salting out" extraction, and precipitation by ethanol (24).

PCR amplification and cloning of rearranged Ig V genes have been described previously (25). Briefly, first-strand cDNA (1–5 μl) was amplified using sense IgV_H gene family-specific primers: V_H1, 5′-GGAATTCATGGACTGGACCTGGAGGGTCTTCT; V_H2, 5′-GGAATTCATGGACATACTTTGTTCCACGCTCC; V_H3, 5′-GAGTTTGGGCTGAGCTGGGACTTTT; V_H4, 5′-ATGAAACACCTGTGGTTCTTCCTCC; V_H5, 5′-GGCTGTTCTCCAAGGAGTC; and V_H6, 5′-GGAATTCATGTCTGTCTCCTTCCTCATCTTCC and antisense C_H constant region primers: μ, 5′-CAAGCTTAAGGAAGTCCTGTGCGAG; γ, 5′-GTAGGACAGC(CT)GGGAAGGTGTGCAC, and α, 5′-CCAAGCTTGAGGCTCAGCGGGAAGACCTT in independent reactions. PCR was performed for 35–45 cycles under standard conditions (25). In some experiments, a V_H3-30 (5′-GGGTTTTCCTCGTTGCTCTTTT) gene-specific primer was used; this strategy allows the amplification of *V_H3-30*, *V_H3-30.3*, and *V_H3-33* genes only.

When genomic DNA was used as template, a first amplification was conducted with the V_H family-specific and a mixture of antisense J_H-specific primers: J_HA, CTGAGGAGACGGTGACCAGGGT; J_HB, CTGAGGAGACAGTGACCAGGGT; J_HC, CTGAGGAGACGGTGACCGTGGT; and J_HD CTGATGAGACGGTGACCATTGT.

To analyze the third complementarity-determining region (CDR) of the H chain variable region gene (HCDR3) lengths, the first PCR products were reamplified using two nested consensus primers, a sense framework region (FR) 3 and an antisense J_H primer (23), and the products were electrophoresed through a 7.5% acrylamide gel and the bands were visualized by a silver staining protocol (Promega, Madison, WI).

PCR specific for clonal sequences

In selected experiments, a strategy was applied to search for a particular V(D)J rearrangement. Primers specific for the CDR2 region of clone 1A-3G7 (5′-TTTCACCTGTCGGCAACG), for the CDR3 region of clone 1A-4G21 (5′-CAGAGGGGGTGGAAGT), and for the CDR3 region of clone 1A-4G29 (5′-GGACTGACTGGGAATGT) were designed. These primers were used in conjunction with the J_H primer in nested PCR and their products were electrophoresed as described above.

cDNA sequencing

First PCR products were purified (Advantage PCR Pure kit; Clontech Laboratories, Palo Alto, CA), and cloned into TOPO TA vector (Invitrogen, Carlsbad, CA), processed using Wizard minipreps (Promega), and sequenced. Sequences were compared with those in the V BASE sequence directory (26) using the MacVector software version 6.0.1 (Eastern Kodak, New Haven, CT). The D segments were assigned to the appropriate family according to the criteria of Klein et al. (27). The intrinsic TAQ error in our system was 0.15%. Sequences are deposited in European Molecular Biology Laboratory (EMBL) under the accession numbers (AJ245201–AJ245361).

Results

Oligoclonal expansions of B cells in the CSF from MS patients

PCR analysis of HCDR3 segments was conducted on the CSF cells from 10 patients with MS using primers specific for the V_H and C_H (μ, γ, and α) genes of Ig molecules. Restricted and dominant (oligoclonal) HCDR3 lengths were identified in all of the CSF samples with each of the V_H family-specific primers, but they were more numerous within the *V_H3* or *V_H4* gene families. Oligoclonal HCDR3 lengths also were detected in the DNA preparations of the same CSF samples, thus excluding that the oligoclonal pattern observed was related to the presence of activated B cells or plasma cells that are enriched in homogeneous RNA. In contrast, only 3 of 10 patients with OND displayed oligoclonal HCDR3 bands. Notably, these three patients had viral encephalitis (patients 6B and 10B, see Table II) or postinfection radiculitis (patient 8B, Table II). Oligoclonal HCDR3 bands were not observed by PCR using cDNA prepared from the PBMC of MS patients or controls (Tables I and II and Fig. 1).

Somatic mutations in the V_H3 and V_H4 genes from the CSF of two MS patients

V_H3 and *V_H4* γ cDNA clones from the CSF of patient 1A ($n = 20$) and patient 2A ($n = 22$) were sequenced (Table III). In patient 1A, certain *V_H3* or *V_H4* genes were predominantly expressed (Table III). Some of the molecular clones were identical, whereas others, such as clones 1A-3G1, 1A-3G4, and 1A-3G8 were related (i.e., they shared the same HCDR3 and differed for a number of point mutations in the V_H gene). In patient 2A, the expansion of *V_H3* and *V_H4* sequences was more heterogeneous, although there were two molecular clones (2A-3G22, 2A-3G26) that carried the same V_H3 gene and shared HCDR3-related sequences. In both patients, the *V_H3* and *V_H4* genes analyzed displayed deviations from the germline genes. In patient 1A, these differences ranged from a minimum

Table II. *Major features of the OND patients*

OND Patients	Clinical			CSF Cells/mm^3	CSF Cells No. PCR[a]	Oligoclonal IgG Bands (IEF)[b]		HCDR3 Band Analysis (PCR)[c]	
	Sex	Age	Diagnosis			CSF	S	CSF	PB
1B	M	26	Viral encephalitis	8	84×10^3	−	−	—	Poly
2B	F	47	Epilepsy	2	26×10^3	−	−	—	Poly
3B	F	74	Transient global amnesia	21	200×10^3	−	−	—	Poly
4B	M	27	Myopathy	1	13×10^3	−	−	—	Poly
5B	F	39	Postinfective myelitis	4	40×10^3	−	−	—	Poly
6B	M	73	Viral encephalitis	22	88×10^3	+	+	Oligo	Poly
7B	F	61	Outcome of polio	1	18×10^3	−	−	—	Poly
8B	F	69	Postinfective myeloradiculitis	69	345×10^3	+	−	Oligo	Poly
9B	F	74	Paralysis 6 c.n.	2	26×10^3	−	−	—	Poly
10B	F	24	Viral meningitis	28	224×10^3	−	−	Oligo	Poly

[a] The total number of cells used for the PCR.
[b] Presence (+) or absence (−) of distinct bands.
[c] Refers to the presence of discrete bands observed in at least two major V_H families (V_H3/V_H4); poly refers to the presence of small, smearing bands; — denotes absence of obvious bands.

of 4.1% to a maximum of 15% (average, 9.2%), and, in patient 2A, these differences were between 1.1 and 10.5% (average, 5.6%).

Clonally related V_H3 sequences in the CSF of MS patients

Among the cDNA clones that shared the same HCDR3 sequence, the *V_H3* or *V_H4* genes were either identical or differed by a few mutations, indicating an ongoing process of intraclonal diversification. To analyze larger numbers of related clones, we designed a strategy of PCR amplification in which primers specific for the *V_H3-30* and *V_H3-30.3* genes were employed in conjunction with γ-chain-specific primers. These primers also could amplify the *V_H3-33* gene. With this method, we isolated 15 of 15 molecular clones from patient 1A that carried the *V_H3-30.3* gene and 15 of 15 clones from patient 2A carrying the *V_H3-30* gene. Thus, despite some degeneracy of the V_H3-30 primers, only molecular clones that harbored the rearranged *V_H3-30.3* (for patient 1A) and *V_H3-30* (for patient 2A) genes most commonly represented in the samples analyzed above were isolated. In patient 1A, 15 of 15 of these molecular clones shared identical or related HCDR3 sequences, whereas 10 of 15 clones from patient 2A had related HCDR3. Fig. 2 reports the all of the clonally related sequences detected in the two patients with the two different primers (i.e., V_H3 and V_H3-30-specific primers). These findings allowed us to depict possible patterns of evolution of each group of related clones (Fig. 3). Notably, mutations of certain codons were repeatedly observed during clonal evolution (see, for example, the replacement Val→Ala at codon 2 in clones 2A-3G1.4, 2A-3G1.14, 2A-3G1.20, 2A-3G12, and 2A-3G1.22 or Gln→Tyr at codon 82 of clones 2A-3G1.13, 2A-3G1.20, 2A-3G1.12, and 2A-3G1.16).

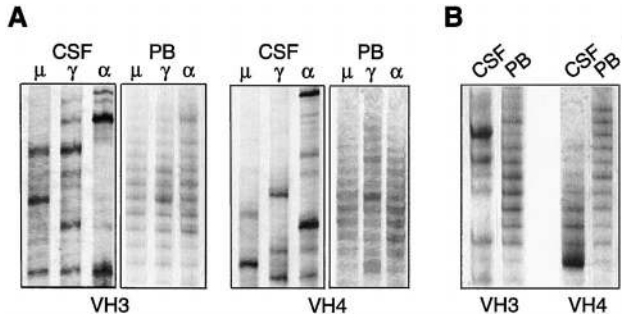

FIGURE 1. PCR analysis of HCDR3 length in CSF cells and in PBMC from MS patients. This 7.5% acrylamide gel shows the relative lengths of the V_H3 and V_H4 CDR3 cDNA (*A*) and DNA (*B*) from the CSF and PBMC of an MS patient (patient 10A). The cDNA was amplified using V_H3 and V_H4 primers along with μ-, γ-, and α-specific primers; the PCR on the DNA was performed by using V_H3- or V_H4-specific primers and J_H primers.

In a subsequent experiment, cDNA from patients 1A and 2A were PCR amplified with the same V_H3-30 primers employed above in conjunction with a μ-chain-specific primer. Twenty molecular clones from patient 1A and 19 from patient 2A were sequenced. None of the clones detected was related to those observed in the γ cDNA since they constantly differed in the HCDR3 sequences. However, in both patients, there were groups of clonally related sequences (one group of two clones from patient 1A, four groups of two clones each from patient 2A) as determined by the HCDR3 identity and the expression of the same V_H3 gene with different patterns of mutations (data not shown; the sequences are available in the EMBL database, accession numbers AJ245273–AJ245311).

Search for the presence in PBMC of the same γ cDNA detected in the CSF cells

In this study, we investigated whether a particular V(D)J sequence (clone 1A-3G7) detected in the γ cDNA from the CSF of patient 1A could also be found in PBMC of the same individual. To this end, two different approaches were used. First, the γ cDNA from PBMC of patient 1A was PCR amplified by using the V_H3-30-specific primer. Among the 20 molecular clones sequenced, none was found to be related to the *V_H3-30*-carrying molecular clones expanded in the CSF of the same patient (data not shown). Second, the V(D)J segment characteristic of the clone (1A-3G7) was amplified from PBMC of the same patient by using a nested PCR methodology. With this method, the first PCR product was reamplified using clone-specific primers (see Fig. 4). As shown in Fig. 4, a distinct band (*lane 3*) was observed by acrylamide gel electrophoresis in the PBMC of patient 1A, which comigrated with both the PCR product of the clone 1A-3G7 and with the PCR product amplified from the CSF of patient 1A (*lanes 1* and *2*). Conversely, no bands were observed in the PBMC or CSF of an unrelated patient amplified as described (Fig. 4, *lanes 4* and *5*). The sequence of the band detected on the PBMC of patient 1A proved to be identical to clone 1A-3G1 (data not shown). The same methodology was employed to search for the sequence of clones 1A-4G21 and 1A-4G29 from the CSF of patient 1A in the PBMC of the same patient. In both cases, no obvious bands were detected.

Table III. *Molecular genetic characteristics of the V_H3 and V_H4 genes in γ cDNA from two MS patients*[a]

Clone[b]	Most Similar Germline V_H Gene[c]	% V_H Gene Difference	Observed R/S CDR	Observed R/S FR	Likely D Segment	J_H	CDR3 Sequence[d]	
Patient 1A								
1A-3G1	3-30.3	14	9/4	12/7	4-17	4b	GDVVTTVTTG	YFDY
1A-3G3	3-30.3	14	9/4	12/7	4-17	4b	GDVVTTVTTG	YFDY
1A-3G4	3-30.3	15	9/4	11/10	4-17	4b	GDVVTTVTTG	YFDY
1A-3G7	3-30.3	15	9/4	11/10	4-17	4b	GDVVTTVTTG	YFDY
1A-3G8	3-30.3	13.5	9/4	12/9	4-17	4b	GDVVTTVTTG	YFDY
1A-3G12	3-30.3	15	9/4	11/10	4-17	4b	GDVVTTVTTG	YFDY
1A-3G13	3-30.3	14	9/4	12/7	4-17	4b	GDVVTTVTTG	YFDY
1A-3G6	3-07	9.4	5/1	6/3	3-3	4b	RQSDDYFWSGYPT	FFDY
1A-3G11	3-07	7	3/1	9/5	3-3	6b	NYFDFWSAYFPLH	YYGMDV
1A-4G21	4-30.2	6.8	3/1	8/7	NA	5b	GGGSGYWSYAN	
1A-4G25	4-30.2	6.4	3/1	8/6	NA	5b	GGGSGYWSYAN	
1A-4G27	4-30.2	6.8	3/1	8/7	NA	5b	GGGSGYWSYAN	
1A-4G33	4-30.2	7.5(SC)			NA	5b	GGGSGYWSYAN	
1A-4G23	4-30.4	4.1	2/1	5/5	3-9	3b	GLTGNV	FDI
1A-4G29	4-30.4	4.1	2/1	5/5	3-9	3b	GLTGNV	FDI
1A-4G34	4-30.4	7.1	8/0	5/5	3-9	3b	GLTGNV	FDI
1A-4G32	4-30.4	4.4	2/2	8/0	3-16	4b	RGVWGSYR	YFDY
1A-4G24	4-31	6.4	4/1	7/4	NA	4b	ESSRSTGHL	DY
1A-4G30	4-31	7.4	7/0	6/6	3-10	5b	DRLWFGDG	GP
1A-4G31	4-31	8.4	7/0	8/3	NA	3b	DGPSTILGVVNA	FDI
Patient 2A								
2A-3G14	3-30.3	9.1	10/0	7/4	5-24	4b	DRELEPTYY	YFDL
2A-3G17	3-07	10.5	5/1	12/9	NA	6b	DRSPPCTM	YGMDV
2A-3G20	3-23	7	6/1	8/2	3-3	5b	DFSVTIFGVGR	FDP
2A-3G21	3-23	8.2	9/0	4/9	3-9	3a	GIIRFLDWLE	DGFDV
2A-3G22	3-30	5.8	4/0	6/3	6-19	6b	DGLGGWFSEGA	YYGLDV
2A-3G26	3-30	5.1	4/3	3/4	6-19	6b	DGLGGWFSEGT	YYGLDV
2A-3G27	3-30	5.8	4/0	6/3	6-19	6b	DGLGGWFSEGA	YYGLDV
2A-3G35	3-30+	6	6/1	6/2	4-23	4b	DGRDFGGYRL	DS
2A-3G36	3-23	4.4	5/2	3/0	3-22	2	DVSYYHDTSPQG	YFDL
2A-3G37	3-23	7	6/3	8/2	NA	4b	DNHYGSSL	DS
2A-3G38	3-07	8	5/3	8/3	NA	1	TNRAA	YFHH
2A-4G15	4-39	8	9/0	6/6	6-19	4b	QSSGWSWA	FDY
2A-4G18	4-30.1	4.7	4/2	3/2	NA	4b	AHGTILPYF	FDY
2A-4G19	4-30.1	3.7	4/0	4/2	3-3	4b	DSRFLEWLL	
2A-4G23	4-39	7.7	10/0	6/6	3-22	3b	RKVTVILLVITEP	DGFDI
2A-4G24	4-34	1.1	1/1	0/0	5-18	3b	EQLWVY	AFDI
2A-4G25	4-30.4	0(SC)	0	0	4-23	3b		
2A-4G32	4-34	1.4	0/0	1/1	5-18	6c	GVRYSFGFKG	YYYYYMDV
2A-4G41	4-04	4.1	3/1	3/3	NA	3b	DLFGNFKS	DSFDI
2A-4G42	4-30.2	3.1	2/2	4/0	4-17	2	IDYGDN	WFFDL
2A-4G44	Tou-4.21	8.6	2/2	11/7	3-10	6b	QRRDYFGSGTLHPTRPR	YYGMDV
2A-4G46	4-04	4.1	3/1	3/3	NA	3b	DLFGNFKS	DSFDI

[a] Sequences are deposited in the EMBL (accession numbers AJ245201–AJ245242).
[b] Clone number denotes the patient (e.g., 1A), followed by number of the V_H family analyzed (e.g., 3). The capital letter (G) indicates the isotype of cDNA and is followed by the corresponding clone number.
[c] Genes identified by two-number code, with the first number indicating the family and the second the relative position in the locus from V_H to J_H.
[d] Amino acids on the left contributed by D segment; those on the right by J_H. NA, not assignable; SC, stop codon.

Collectively, these data demonstrate an imbalanced expression of B cell clones between CSF and PBL.

Analyses of V_H3 γ cDNA from the CSF of an OND patient

In these studies, the V_H3 γ transcripts from the CSF of an OND patient (10B) that displayed oligoclonal PCR bands (Table II) were sequenced. All of the V_H3 genes ($n = 16$) analyzed showed significant deviations from the germline (average mutation frequency, 7.1%). Among the sequences, there were four groups, of two clones each, that contained repeated sequences (i.e., identical HCDR3 and identical pattern of mutations on the V_H gene). One group of these clones carried the *V_H3-30* and another the *V_H3-33* gene (Table IV). Therefore, to determine whether intraclonal diversity had developed among these clones, we employed the same strategy of amplification with V_H3-30-specific primers used above. Fourteen molecular clones were isolated and sequenced. Notably, although there was evidence for amplification of identical clones expressing the *V_H3-33* gene (see Table IV), there were no instances of intraclonal diversification.

Discussion

Analyses of HCDR3 length revealed oligoclonal bands in the CSF cells from 10 of 10 MS patients. Detection of oligoclonal bands by PCR is not unusual when low cell concentrations are employed and we have observed those bands in the PBMC of normal individuals by diluting out their B cells (data not shown). However, when artifacts related to the presence of restricted B cells or plasma cells or to the preferential amplification of certain V genes or gene families are ruled out, the observation of HCDR3 gene segments of different lengths is taken as broad evidence for the

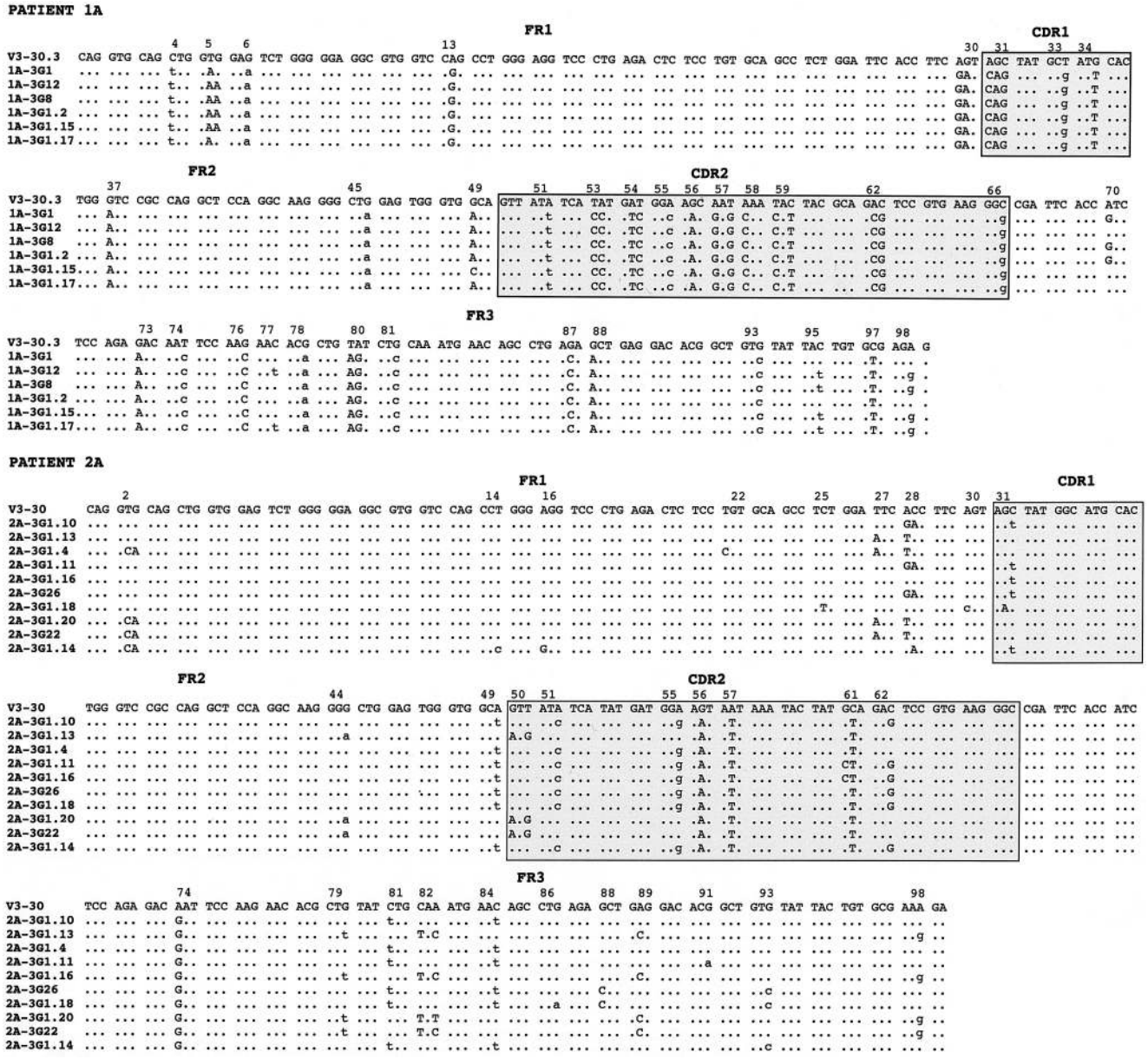

FIGURE 2. Intraclonal diversification of γ V_H3 transcripts in the CSF from two MS patients. Clonally related sequences from patients 1A and 2A amplified with V_H3 or V_H3-30 primers were pooled, aligned, and compared with the most homologous germline V_H3 sequences (V_H3-30.3 for patient 1A and V_H3-30 for patient 2A, respectively). The dots denote germline identity. Capital and lower case letters represent replacement and silent mutations, respectively.

nonrandom distribution of B cells, most likely consequent to accumulation of certain clonal progenies. Indeed, such accumulations are observed in the synovial tissues of rheumatoid arthritis patients, where an active B cell response, causing a compartmentalized B cell proliferation, is occurring (28–33). Notably, accumulation of oligoclonal bands was also revealed in 3 of 10 OND patients, possibly indicating that nonrandom distribution of B cell clones in the CSF is not a distinctive feature of MS, but may characterize a variety of infectious or autoimmune disorders. In connection with this, it is of note that two of three OND patients studied had oligoclonal IgG in the CSF detected by IEF (see Table II). These observations suggest a correlation between the presence of accumulations of B cell clones detected by PCR and oligoclonal IgG bands. Studies are currently in progress to explore whether PCR can be an additional or even an alternative test to IEF.

The striking finding that emerged from these studies was that in the CSF of two patients with different clinical forms of MS, there were clonally related sequences that differed from each other by the accumulation of distinct point mutations. The use of a PCR strategy designed to amplify gene-specific sequences verified the presence of clonal lineages. The V_H genes in γ cDNA were highly mutated and mutations of certain codons were repeatedly observed during clonal evolution, thus reinforcing the notion that a strong pressure was imposed upon the proliferating B cells by the stimulating/selecting Ag(s). In connection with this, it is perhaps worth mentioning that studies on CSF samples taken from the same patient at 1-year intervals have demonstrated the presence of the same V(D)J sequences, possibly reinforcing the hypothesis of the presence of a continuous selective stimulation. Longitudinal studies are currently in progress.

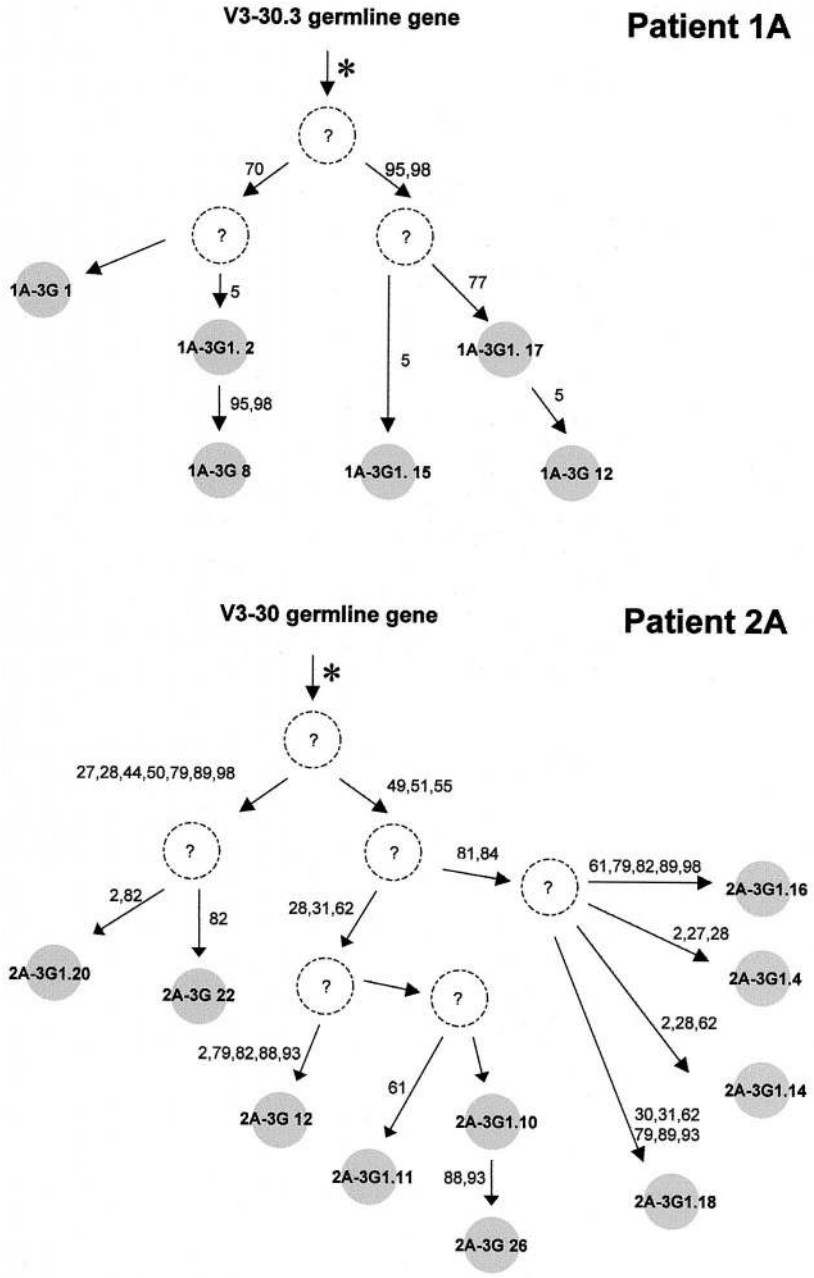

FIGURE 3. Patterns of clonal evolution in MS. The diversification of two clones, one from patient 1A and one from patient 2A, respectively, is depicted. The clone from patient 1A expressed the V_H3-30.3 gene and that from patient 2A the V_H3-30 gene. ? indicates all hypothetical intermediate sequences. ∗Ancestor gene differing from the germline gene by 39 substitutions (patient 1A) or 4 substitutions (patient 2A). Numbers beside the arrows refer to the amino acid residues where the mutations are found.

The search for clonally related sequences was also extended to the μ cDNA. Although these studies demonstrated the presence of clonally related V gene sequences also in μ cDNA, they failed to reveal sequences shared by the μ and γ cDNA from the same patient, suggesting that isotype switching was a rare event.

Despite gene-specific PCR strategies, clonal diversification was not demonstrated in the CSF of one patient with viral meningitis. Although these studies revealed the presence of V_H genes with abundant point mutations, they failed to demonstrate clonal lineages, suggesting that clonal diversification is more frequent in MS and may represent a peculiar characteristic of this and certain other demyelineating diseases.

The presence of clonally related sequences is a relatively common finding in B cells purified from germinal centers, but it is uncommon for B cells of other subsets (34, 35). In this respect, the B cells from the CSF of MS patients resemble those developing in the germinal centers in the course of an immune response (34). Notably, the majority of clones isolated from MS patients presented evidence for Ag stimulation and not for Ag selection, at least based upon calculations according to the replacement (R): silent (S) ratio in the CDR vs FR (Table III) or to the Chang-Casali algorithm (data not shown) (36–38). However, the R:S ratio calculated in the FR (1.43 for patient 1A and 1.53 for patient 2A) suggested some counter selection by the stimulating Ag. Accumulations of clonally related B cells have been described in tissues that are presumptive targets of autoimmune reactions such as the synovia of patients with rheumatoid arthritis (28–31) or the salivary glands of patients with Sjogren's syndrome (39, 40). In the case of MS, there are many parameters that need to be clarified. These include the site where B cells are first stimulated, the potential mechanism of subsequent stimulation/selection, and the mode of migration of activated B cells to and from the CNS, in addition to the nature of the stimulating autoantigens (41–43). Notably, it is not known what are the sites of antigenic stimulation/selection in the CNS that are possibly characterized by accumulation of follicular dendritic cells.

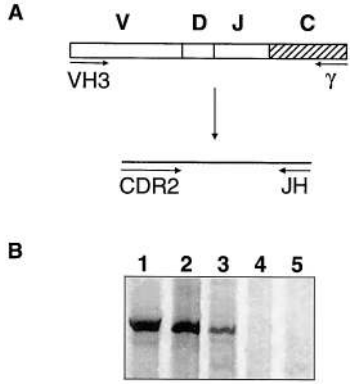

FIGURE 4. Detection of a V(D)J sequence characteristic of CSF cells in PBMC of patient 1A. *A*, Schematic diagram showing the PCR procedure used for the search of the V(D)J sequence characteristic clone 1A-3G7 found in the CSF cells of patient 1A. Total cDNA was amplified first using V_H3- and γ-specific primers and then reamplified by using clone-specific CDR2 and J_H primers. *B*, 7.5% acrylamide gel electrophoresis analysis of amplified V(D)J cDNA. *Lane 1*, molecular clone 1A-3G7 isolated from the CSF of patient 1A; *lane 2*, CSF of patient 1A; *lane 3*, PBMC of patient 1A; *lanes 4* and *5*, CSF and PBMC, respectively, of an unrelated patient (patient 8A).

Whatever the fine pathogenic mechanisms may be, our data indicate that in the CSF of MS patients there may be an intensive antigenic stimulation, possibly by a relatively restricted number of Ags. In connection with this, Owens et al. (44) found accumulations of related *V_H4*-expressing clones in different areas of an acute MS brain. These and other observations demonstrating a restricted pattern of Ig mRNA within the plaque lesions (45) are consistent with the present description of oligoclonal B cell expansion and diversification in the CSF. Recently, using a RT-PCR methodology with primers specific for the V(D)J segments, Qin et al. (46) demonstrated oligoclonal and sometimes monoclonal B cell expansions in the CSF of MS patients. The expanded clones were somatically mutated with a distribution of mutations suggesting Ag selection. However, the presence of clonally related sequences was not detected. An explanation for these discrepancies is not easy, particularly in view of the many methodological differences, but is likely to be somehow related to the lower sensitivity of the RT-PCR method, the different primers, and the more limited number of molecular clones sequenced by Qin et al. (46).

The search for dominant HCDR3 cDNA lengths and V(D)J sequences in the PBMC corresponding to those detected in the CSF was virtually negative. These findings support the notion that in MS there is an expansion of B cells possibly occurring within the CSF. Alternatively, the B cells from the same clones detected in the CSF may preferentially home and possibly expand at certain particular sites, like cervical lymph nodes, as it has been proposed (41, 42, 47–49). The available RT-PCR methodology may now permit to explore the possibilities and to trace relationships between B lymphocytes in the CSF and those found at other anatomical sites.

Acknowledgments

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Table IV. *Molecular genetic characteristics of the V_H3 gene in γ chain cDNA from an OND patient (10B)*[a]

Clone	Most Similar Germline V_H Gene	% V_H Gene Difference	Observed R/S		Likely D Segment	J_H	CDR3 Sequence	
			CDR	FR				
Group 1								
10B-3G1	3-23	10.5	5/4	8/10	3-9	4b	VVRPHYDIFSNYYGID	
10B-3G2	3-15+	10.6	2/4	11/10	NA	5b	VRGREFLR	
10B-3G5	3-15+	10.6	2/4	11/10	NA	5b	VRGREFLR	
10B-3G3	3-30	2.7	4/1	4/3	2-15	4b	DGTVLVVAATAYGAA	HLDQ
10B-3G8	3-30	2.7	4/1	4/3	2-15	4b	DGTVLVVAATAYGAA	HLDQ
10B-3G4	3-07	4.3	3/0	3/3	3-9	6b	DLYYDFLTGYGSDN	GMDV
10B-3G6	3-33	4.3	4/1	2/3	NA	4b	EGQIVAYY	FDY
10B-3G7	3-07	20	11/2	20/12	NA	5b	GDGWLVDL	
10B-3G10	3-15+	5.9	3/3	5/6	3-10	4b	GFMVRGTITTN	HDY
10B-3G11	3-09	6.4	3/1	9/2	NA	4b	TLVTTKPFG	Y
10B-3G13	3-07	8.4	6/1	7/4	NA	5b	GRPRFDR	
10B-3G14	3-23	7.8	6/1	7/4	3-10	4b	TGASGL	FDY
10B-3G18	3-23	7.8	6/1	7/4	3-10	4b	TGASGL	FDY
10B-3G15	3-33	2.3	2/0	2/3	4-17	4b	DPVYGDLYY	FDY
10B-3G16	3-33	1.3	1/1	2/0	NA	4b	DSDPGVFDSTW	FDY
10B-3G20	3-33	1.3	1/1	2/0	NA	4b	DSDPGVFDSTW	FDY
Group 2								
10B-3G1.1	3-33	4.3	4/1	2/3	NA	4b	EGQIVAYY	FDY
10B-3G1.2	3-33	4.3	4/1	2/3	NA	4b	EGQIVAYY	FDY
10B-3G1.3	3-33	4.3	4/1	2/3	NA	4b	EGQIVAYY	FDY
10B-3G1.4	3-33	4.3	4/1	2/3	NA	4b	EGQIVAYY	FDY
10B-3G1.7	3-33	4.3	4/1	2/3	NA	4b	EGQIVAYY	FDY
10B-3G1.8	3-33	4.3	4/1	2/3	NA	4b	EGQIVAYY	FDY
10B-3G1.12	3-33	4.3	4/1	2/3	NA	4b	EGQIVAYY	FDY
10B-3G1.14	3-33	4.3	4/1	2/3	NA	4b	EGQIVAYY	FDY
10B-3G1.5	3-07	20	11/2	20/12	NA	5b	GDGWLVDL	
10B-3G1.6	3-33	2.3	2/0	2/3	4-17	4b	DPVYGDLYY	FDY
10B-3G1.9	3-33	7.4	4/1	7/7	NA	4b	EKGNWLVWGTKP	FDY
10B-3G1.10	3-33	7.4	4/1	7/7	NA	4b	EKGNWLVWGTKP	FDY
10B-3G1.11	3-33	1.3	1/1	2/0	NA	4b	DSDPGVFDSTW	FDY
10B-3G1.15	3-33	1.3	1/1	2/0	NA	4b	DSDPGVFDSTW	FDY

[a] See legend to Table 2. Sequences are deposited in EMBL under accession numbers AJ245312–AJ245341. Group 1 and Group 2 denote clones obtained following an amplification with either V_H3-specific or with V_H3-30-specific primers, respectively, in conjunction with γ-specific primers.

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