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Intrathecal B-Cell Clonal Expansion, an Early Sign of Humoral Immunity, in the Cerebrospinal Fluid of Patients with Clinically Isolated Syndrome Suggestive of Multiple Sclerosis

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SUMMARY: The development of somatically mutated memory and plasma B cells is a consequence of T cell-dependent antigen-challenged humoral immunity. To investigate the role of B cell-mediated humoral immunity in the initiation and evolution of multiple sclerosis (MS), we analyzed Ig variable heavy chain genes of intrathecal B cells derived from patients with a first clinical manifestation suggestive of MS. Sequences of Ig variable regions showed that B cells in the cerebrospinal fluid from most of these patients were clonally expanded and carried somatic hypermutated variable heavy chain genes. The mutations showed a high replacement-to-silent ratio and were distributed in a way suggesting that these clonally expanded B cells had been positively selected through their antigen receptor. In comparison, intrathecal B-cell clonal expansion often precedes both oligoclonal IgG bands and multiple magnetic resonance imaging lesions. Clinical follow-up study showed that patients with clonally expanded intrathecal B cells had a high rate of conversion to clinically definite MS. The findings provide direct evidence of recruitment of germinal center differentiated B lymphocytes into the central nervous system during the initiation of MS. These results indicate B cell-mediated immune response in the cerebrospinal fluid is an early event of inflammatory reaction in the central nervous system of MS. This procedure also provides a more sensitive method to evaluate the association of humoral immunity in the evolution of MS. (*Lab Invest 2003, 83:1081–1088*).

Multiple sclerosis (MS) is a chronic (auto)immune inflammatory disease that causes disability due to immune-mediated damage of myelin sheath and axons (Adams, 1983; Smith et al, 1993). Within demyelinating lesions, there is an accumulation of lymphoid and myeloid cells, including T and B lymphocytes, monocytes, and macrophages (Compston et al, 1989; Hafler et al, 1985; Wucherpfennig et al, 1997). The infiltration of B cells and deposition of antibody-complement complexes indicate that humoral immunity facilitates inflammation and accelerates damage to myelin sheaths and axons (Cross et al, 2001; Genain et al, 1999; Lucchinetti et al, 2000).

Several recent studies indicate that permanent tissue damage occurs early in the course of MS (Ganter et al, 1999; Trapp et al, 1998). In most cases of MS, the first clinical manifestation is clinically isolated syndromes (CIS) involving optic neuritis, transverse myelitis, or a brain stem/spinal cord syndrome. The role of an antigen-driven immune response in the initiation and the development of MS at this stage is still unclear. Based on experiments in rodents and humans, somatic hypermutation of B-cell Ig variable region genes occurs during B-cell differentiation which, when coupled to T cell-dependent antigen selection in germinal centers (GCs), results in affinity maturation, immune memory, and B-cell clonal selection (Archelos et al, 2000; Baranzini et al, 1999; Colombo et al, 2000; Correale and de Los Milagros Bassani Molinas, 2002; Cross, 2000; Owens et al, 1998; Qin et al, 1998).

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Early studies have shown that clonal expansion is based on immune memory, which results in an enhanced response on restimulation with the same antigen. Memory is generated by an increase in affinity of B-cell clones for the antigen resulting from somatic mutation of the Ig V gene, with the B cells undergoing a Darwinian clonal positive selection process based on their affinity for antigen held on follicular dendritic cells in GCs. High-affinity mutants survive, whereas low-affinity ones cannot bind antigen and die by apoptosis (Hollowood and Macartney, 1991; Liu et al, 1989). Under persistent antigen stimulation, memory B-cell clones undergo massive proliferation and dominate a specific B cell-mediated humoral immune response throughout the lifetime of the organism (Gray and Matzinger, 1991; Gray and Skarvall, 1988; Slifka et al, 1998).

In the present study, we have studied B-cell clonal expansion and somatic hypermutation of Ig-variable heavy chain (V_H) genes expressed by intrathecal B cells from patients with a first clinical manifestation suggestive of MS. We show that antigen-driven Ig-V_H gene somatic hypermutation and B-cell clonal expansion are characteristic of the adaptive immune response in the cerebrospinal fluid (CSF) of a majority of these patients. Patients with adaptive immune response in the CSF showed a high risk of developing clinically definite MS (CDMS).

Results

Clinical Assessment

To investigate the nature of the B-cell response in the central nervous system (CNS) in MS, analyses of B-cell clonality of the CSF cells and antigen-driven Ig-V_H gene somatic hypermutation were performed by RT-PCR and sequencing techniques. We studied a total of 48 individuals, 16 with a CIS suggestive of MS and 32 with other neurologic diseases (OND). The clinical features, laboratory, and brain magnetic resonance imaging (MRI) findings of these CIS patients are presented in Table 1. At the time of testing for B-cell clonal expansion and Ig-V_H gene somatic hypermutation, laboratory examination showed that, of the 16 patients with a CIS suggestive of MS, 7 had no CSF oligoclonal bands (OCBs), 5 had no lesions on MRI, 3 had one spinal lesion, and 8 had $\geq$ three lesions.

CSF B Cell Clonality

Figure 1 shows PCR amplification of the Ig complementarity-determining region 3 (CDR3) genes from intrathecal B cells. PCR bands indicating CDR3 Ig-V_H genes were seen in 13 of 16 patients with a CIS suggestive of MS (Fig. 1A) and 3 of the 32 OND (Fig. 1B). Faint bands were seen in 1 of 16 CIS cases and in 3 OND cases. Table 2 shows amino acid sequences of CDR3 genes. Sequence analysis showed B-cell monoclonal and dual clonal expansion in 12 cases and polyclonal expansion in 1 case with CIS suggestive of MS. CSF B-cell clonal (1 case) and polyclonal (2 cases) expansion was seen in 3 of 32 OND cases. In identical clones, the V_H-N-D-N-J_H regions were identical both in length and sequence. The different sequences of CDR3 found in polyclonal B cells (Case 6) showed clear differences both in length and sequence. In comparison with laboratory and MRI results, 5 of 13 cases with intrathecal clonal and polyclonal B-cell expansion had no CSF OCB and 6 cases had no disseminated (more than two) MRI lesions. For three CIS cases with faint or no PCR bands, laboratory findings showed that all three cases had no CSF OCB and one case had one spinal cord lesion (Table 1)

Table 1. Summary of the Clinical and Laboratory Information from Patients with CIS

Samples	Sex	Age at onset	MRI lesions at onset	CSF		EDSS
				OCB	Cell number 10^4	
1	F	28	0	+	16	3.0
2	F	48	>3	−	9	0.0
3	F	41	0	−	20	3.0
4	F	24	>3	+	4	2.5
5	F	21	1S	+	2	2.0
6	F	29	3	−	10	2.0
7	M	35	>3	+	4	2.0
8	M	45	>3	+	1	1.0
9	M	22	>3	+	2	3.0
10	F	26	0	−	4	0.0
11	F	24	0	−	1	1.0
12	F	34	>3	+	5	6.0
13	F	35	>3	+	2	1.0
14	F	34	1S	+	3	2.5
15	F	39	1S	−	1	1.0
16	F	29	0	−	3	0.0

Summary of CIS patients in this study. MRI, brain and spinal cord magnetic resonance imaging; OCB, oligoclonal bands; Cell number, total number of white blood cells in the specimen (2–8 ml); EDSS, expanded disability status score; S, spinal cord.

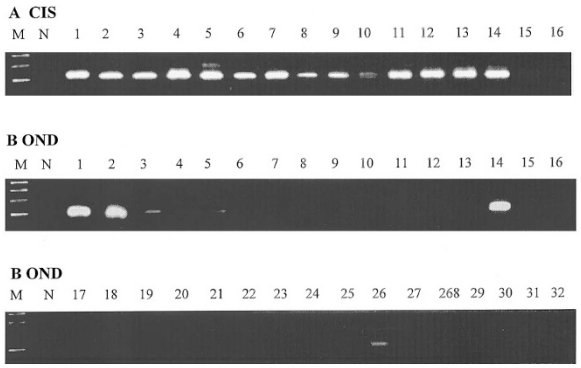

Figure 1.

Ethidium bromide-stained 2% agarose gel of complementarity-determining region 3 (CDR3) PCR products. Lane M: molecular weight markers (size in bp); lane N: negative control. A, Lanes 1 to16: cerebrospinal fluid (CSF) samples from patients with a clinically isolated syndrome (CIS) suggestive of multiple sclerosis (MS). B, Lanes 1 to 32: CSF from patients with other neurologic diseases (OND).

A strong Ig-V_H gene PCR band was found in the CSF of 3 of 32 OND patients: 2 of them (1 polyclonal and 1 monoclonal intrathecal B-cell expansion) had acute disseminated encephalomyelitis and 1 case with herpes zoster encephalomyelitis had polyclonal intrathecal B-cell expansion.

V_H Family Utilization in Intrathecal B-Cell Clones

To determine V_H family usage, sequences of V_H genes expressed by intrathecal clonally expanded B cells were aligned to the closest germline V_H genes (Table 3). The V_H3 family was significantly overrepresented with 6 of 14 clones (including dual clones from Cases 1 and 7) derived from the four members V_H3–15 (Cases 5 and 14), V_H3–48 (Case 7), V_H3–11 (Case 11), and V_H3–30 (Cases 12 and 13). Four of 12 clones used the V_H4 family, derived from the three members V_H4–59 (Case 2), V_H4–39 (Cases 3 and 8), and V_H4-DP70. The other 3 clones used the V_H1 family, derived from the three members V_H1–8 and V_H1–46 (Case 1) and V_H1–69 (Case 7) (Altschul et al, 1997; Matsuda et al, 1998). To control the primer bias, we compared these V_H families with those obtained with polyclonal expanded intrathecal B cells using the same V_H primers, in which no dominant V_H3 and V_H4 families were obtained (Table 3, Case 6).

Somatic Hypermutations

To study the differentiation pathway of intrathecal clonally expanded B cells, somatic hypermutation of Ig-V_H genes expressed by clonally expanded intrathecal B cells was analyzed. The differences in nucleotide sequence when compared with the closest known germline V_H genes show that the combined replacement (R) to silence (S) ratios for the framework region (FRs) and CDR domains are derived from the sum of all mutated codons in the V_H sequences of the intrathecal B cells assignable to the germline gene segment (Table 3). For 12 cases of CIS, the average replacement-to-silent ratio (R:S) in the FRs was 1.6, whereas that in CDR1 and CDR2 was 5.2. They were significantly higher and lower 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 (Jukes and King, 1979). A higher CDR R:S mutation ratio reflects positive selective pressure applied by an antigen to gene products that come into close contact with antigen. A lower FR R:S mutation ratio reflects the negative pressure for mutant selection applied to structural components that need to be conserved. Some of this sequence variation may be attributable to infidelity of *Taq* polymerase, which has been reported to result in an error rate of 1/5000 to 1/9000 errors per base polymerized (Oste, 1988; Tindall and Kunkel, 1988).

Clinical Follow-Up

To study the predictive value of clonal expansion of hypermutated intrathecal B cells for the development of CDMS, patients were followed up clinically. After 1 to 6 years of follow-up, 10 of the 13 patients with clonal expansion of intrathecal B cells developed to relapsing-remitting MS (RRMS) (Table 4). Six of these 10 cases had disseminated ($\geq$3) MRI lesions, 4 cases had no disseminated ($\leq$1) MRI lesion, and 4 cases had no CSF OCB (Table 4).

Discussion

The goal of the present study was to clarify the importance of B cell-mediated humoral immunity in the initiation and development of MS. We show here that B cells in the CSF of patients with a first clinical manifestation of suggestive MS, with or without disseminated MRI lesions or OCBs, were clonally expanded and carried somatic hypermutated V_H genes. The infiltration of clonal B cells preceded the appearance of both MRI lesions and OCBs. Clinical follow-up showed that patients with clonal expansion of hypermutated intrathecal B cells demonstrated a high risk of developing CDMS.

In most cases of MS, the first clinical episode is a CIS. Progress in understanding and detecting the immune mechanisms in CIS suggestive of MS has been hampered by the lack of sensitive systems to identify an antigen-driven adaptive immune response in the CNS. Several recent studies have demonstrated that permanent tissue damage occurs early in the course of MS (Bruck et al, 1995; Ferguson et al, 1997; Ganter et al, 1999; Trapp et al, 1998). The presence of OCB in the CSF, an early sign of immune response in the CNS, as demonstrated by isoelectric focusing techniques is seen in approximately 50% patients at this stage (Avasarala et al, 2001; Noort and Holland, 1999). The ability to recognize an autoimmune reaction in the CNS during the early stages of disease is important to understand the immune pathogenesis in the initiation of MS, to establish a working diagnosis, and to guide an early treatment for delaying or preventing the development of MS.

Table 2. CDR3 Amino Acid Sequences of Dominant Clonally Expanded CSF B Cells

	Identical colonies	V_H	N D N	J_H
CIS				
1	4/8	CAR	GPRND	YWFDRWG (J_H5)
	3/8	CAR	DVGELVGG	NWFDPWG (J_H5)
2	6/7	CAS	SGGSGEYGK	FDYWG (J_H4)
3	5/6	CAR	HHRDHIVVVTAP	FDYWG (J_H4)
4	7/14	CAN	ERVEGGVF	GMDVWG (J_H6)
5	4/7	CST	DVR	FULLG (J_H5)
6	Polyclonal			
	1/10	CAR	HGWLYNSGN	FDSWG (J_H4)
	1/10	CAK	VRYYDP	LDYWG (J_H4)
	1/10	RAR	DLGYSYGF	YGMDVWG (J_H6)
	1/10	CAK	EDHSSGL	HFDNWG (J_H4)
	1/10	CAR	DLNDDFGNYYSTN	YYHYGMDVWG (J_H6)
	1/10	CVR	EDLVVVPAASNL	FDYWG (J_H4)
	1/10	CAR	GGVVPTAML	YWG (J_H4)
	1/10	CAR	HGWFYNSGN	FDSWG (J_H4)
	1/10	CAR	RDCGSMSCPF	DHWG (J_H5)
	1/10	CAR	VGYCTNGVCYVG	DYYYGMDVWG (J_H6)
7	3/6	CAR	SARYYDTSGHYTP	FDYWG (J_H4)
	3/6	CAR	TYSNRW	HYWG (J_H4)
8	3/5	CAK	TFPNSYW	YYYGMDVWG (J_H6)
9	4/6	CAR	GKAVTGLWPGHGWTY	YFDYWG (J_H4)
10	Ig-V_H gene undetectable			
11	5/6	CAR	EGIKIWSQFSPGDDY	YGMDVWG (J_H6)
12	4/6	CAR	DLYLVRGH	FDSWG (J_H4)
13	4/6	CAR	EFRSVKANSADNRR	TDYWG (J_H4)
14	4/5	CAK	DMGYHYASDSS	YFDSWG (J_H4)
15	Ig-V_H gene undetectable			
16	Ig-V_H gene undetectable			
OND				
1	Polyclonal			
2	Polyclonal			
3 to 13	Ig-V_H gene undetectable			
14	5/6	CAK	TNRYSSGPF	YYYGMDVWG (J_H6)
15 to 32	Ig-V_H gene undetectable			

Predominant CDR3 region amino acid sequence of CSF B cells from patients with CIS or OND. The sequences of the CDR3 region are grouped and subdivided into V_H, N, D, N, and J_H regions. The name of the germline D and J_H genes showing maximal homology to the segments used in the VDJ joining is shown in parenthesis at the appropriate position.

This study in CIS patients supports the early involvement of B cell-mediated humoral immunity in MS. Sequences of the CDR3 gene fragment of CSF B cells were analyzed to determine the clonality of these B cells. The criteria for establishing clonal relatedness were identification of the somatically formed V_H-D_H, D_H-J_H genes and the use of the same sets of V_H D_H J_H genes. Using this approach, we have provided the first evidence for B-cell clonal expansion in the CSF of a majority of these patients (81%), which is significantly higher than the positive rate in the control OND group (3%, $p < 0.01$). The B-cell clonal expansion rate (81%) is also much higher than the OCB rate (50%) and the MRI rate with no or less than two lesions (50%) in the same group of patients. The predominant B-cell clonal expansion in the CSF seems to commonly precede both oligoclonal banding and MRI disseminated lesions. Demonstration of CSF dominant B-cell expansion is a sensitive test for the early assessment of an adaptive immunity in the CNS of MS.

Unlike genes of T cell receptors, the rearranged Ig variable genes in mature B cells can mutate. In T cell-dependent antibody responses, antigen-specific B cells undergo rapid and extensive clonal expansion in GCs. T–B cell interaction through CD40 on B cells and CD40 ligand on T cells and cytokines secreted by Th2 cells, such as IL-2, IL-4, and IL-10 are required for GC formation (Macklin et al, 1983; Rissoan et al, 1999). GCs play a critical role in the generation of high-affinity humoral immune response via Ig gene somatic hypermutation. Somatic hypermutation occurs in the variable regions of Ig genes, which often show a marked accumulation of replacement mutations in their CDRs (Griffiths et al, 1984; Nossal, 1992; Siekevitz et al, 1987). CDRs are thought to provide the antigen-binding site (Shlomchik et al, 1987). Cluster-

Table 3. Differences in Nucleotide Sequences in the Ig-V_H Genes of Dominant Clonally Expanded CSF Cells Derived from Patients with CIS

Case	V_H gene	Percent homology	FRs R:S	CDRs R:S	Nucleotides		Total		R:S	
					Insert	Delete	FRs	CDRs	FRs	CDRs
1	V_H1-8	96	3/3	3/0			6	3	1.0	3.0
	V_H1-46	91	7/5	9/1			12	10	1.4	9.0
2	V_H4-59	96	2/7	6/2		6	9	8	0.5	3.0
3	V_H4-39	96	3/2	8/0	6		5	8	1.5	8.0
4	V_H4-DP70	93	9/7	5/1			16	6	1.3	5.0
5	V_H3-15	99	2/0	0/0			2	0	2.0	0.0
6	Polyclonal									
	V_H4-31	90	10/11	6/1			21	7	0.9	6.0
	V_H3-30	100								
	V_H1-69	95	5/2	5/1			7	6	2.5	5.0
	V_H5-51	97	5/0	1/1			5	2	5.0	1.0
	V_H4-31	89	15/9	7/0			24	7	1.7	7.0
	V_H3-11	93	9/4	8/0			13	8	2.2	8.0
	V_H4-39	89	14/9	5/2			23	7	1.6	2.5
	V_H5-51	92	7/5	9/2			12	11	1.4	4.5
	V_H1-69	92	9/3	9/1			12	10	3.0	9.0
	V_H1-69	97	6/1	2/0			7	2	6.0	2.0
7	V_H1-69	90	13/5	9/2			18	11	2.6	4.5
	V_H3-48	92	8/7	7/1			15	8	1.1	7.0
8	V_H4-39	95	6/6	4/0			12	4	1.0	4.0
9	V_H4-49	95	7/4	3/1			11	4	1.75	3.0
10	Ig-V_H gene undetectable									
11	V_H3-11	89	14/7	7/4			21	11	2.0	1.7
12	V_H3-30	93	7/4	6/1			11	7	1.7	6.0
13	V_H3-30	91	11/4	11/0			15	11	2.7	11.0
14	V_H3-15	91	10/5	7/1			15	8	2.0	7.0
15	Ig-V_H gene undetectable									
16	Ig-V_H gene undetectable									

Differences in nucleotide sequences in the V_H genes of dominant clonally expanded CSF cells. FRs, framework regions; CDRs, complementarity-determining regions (including CDR1 and CDR2); R:S, replacement (R) to silence (S) ratios.

ing of R mutations in CDRs has been used as an indicator of T cell-dependent, antigen-driven B-cell immunity (Bahler and Levy, 1992; Qin et al, 1995). The positively selected CSF B-cell clones bore somatic mutations, which were highly concentrated in the CDR or FR regions, with a clustering of replacement mutations and insert codons in the CDRs but only a few in the FRs. This is a characteristic distribution pattern indicating that these B-cell clones have undergone a GC differentiation pathway. Somatic mutation of V genes can increase the ability of the surface Ig receptors to bind antigen, thereby giving the B-cell clone a growth advantage over other B cells that cannot respond to the antigen. Studies of the functional influence of these mutations have shown that the antigen-binding site (CDRs) depends on just 5 to 19 amino acids. A mutation that changes only one amino acid can increase or decrease the antibody's affinity 10-fold or change the antibody's specificity (Allen et al, 1987, 1988). We have demonstrated that in MS, somatic mutations occur mainly in the CDRs with a high R:S ratio and were distributed in a way suggesting that these B cells had been positively selected through their antigen receptor in the GCs of secondary lymphoid tissues and migrated into the CNS (Qin et al, 1998). Other groups have confirmed this observation with samples from CSF and plaques/lesions of MS (Baranzini et al, 1999; Colombo et al, 2000; Owens et al, 1998).

More important, our results show that development of intrathecal B-cell clonal expansion in patients with CIS demonstrates similar characteristics to its development in patients with relapsing-remitting and chronic-progressive MS: (1) it is antigen-driven; (2) there is somatic hypermutation, and (3) there is an involvement of T helper cells. These findings indicate that an antigen-driven adaptive immunity, which has been considered to play an important role in lesion formation, occurred in the CNS of a majority of patients with CIS suggestive of MS. Data further confirm the significance of intrathecal B-cell clonal expansion in both clinic and pathobiology of MS. Analysis of CSF B-cell clonality can provide an objective assessment of an autoimmune process in the evolution of MS. The findings indicate that an ongoing destructive B cell-mediated humoral immunity is occurring in the CNS at this early stage of patients with CIS and is sustained throughout the development to CDMS.

Table 4. Correlation of Intrathecal B Cell Clonal Expansion with Laboratory Information and Conversion of CDMS

Samples	B cell clonality	V_H gene mutations	MRI lesions at onset	OCB	Develop to CDMS (Poser's)	Clinical follow-up (mo)	Current diagnosis
1	Monoclonal	9	0	+	Yes	3	RRMS
2	Monoclonal	22	>3	−	Yes	22	RRMS
3	Monoclonal	25	0	−	Yes	18	RRMS
4	Dual-clonal	22	>3	+	Yes	6	RRMS
5	Monoclonal	2	1S	+	Yes	8	RRMS
6	Polyclonal		3	−	Yes	10	RRMS
7	Dual-clonal	29	>3	+	No	14	Single attack
8	Monoclonal	16	>3	+	No	15	Single attack
9	Monoclonal	15	>3	+	Yes	24	RRMS
10	V_H gene UD	UD	0	−	No	68	Single attack
11	Monoclonal	32	0	−	Yes	17	RRMS
12	Monoclonal	18	>3	+	Yes	3	RRMS
13	Monoclonal	26	>3	+	Yes	14	RRMS
14	Monoclonal	23	1S	+	No	21	Single attack
15	V_H gene UD	UD	1S	−	No	72	Single attack
16	V_H gene UD	UD	0	−	No	50	Single attack

Correlation of intrathecal B-cell clonal expansion with laboratory information and conversion of Clinically definite MS (CDMS). UD, undetectable; Poser's, Poser's diagnostic criteria.

In summary, our data points to an important role for B cell-mediated humoral immune response in the initiation and the development of MS. Our data also indicate the value of the detection of clonal expansion of somatic hypermutated intrathecal B cells at presentation as a predictive and prognostic indicator in patients presenting with a first CIS suggestive of MS.

Materials and Methods

Patients

Based upon published criteria (Lublin and Reingold, 1996; Poser et al, 1983), 16 patients with a CIS suggestive of MS and 32 patients with OND were selected. The study was performed before recent publication of more stringent diagnostic criteria (McDonald et al, 2001). The 16 patients with a CIS suggestive of MS consisted of 12 women and 4 men with a mean age of 32 ± 8 (range, 21–48) years and a mean expanded disability status score of 2.0 ± 2.0. None of the patients had received glucocorticoids or immunosuppressive treatment in the preceding 6 months (Table 1).

Preparation of cDNA and DNA

Total RNA was extracted from 1 to 20 × 10^4 CSF cells isolated from 2 to 8 ml of CSF using an RNeasy kit (QIAGEN Inc., Chatsworth, California). First-strand cDNA was synthesized using oligo d(T) as primer and avian myeloblastosis virus reverse transcriptase. Ig-V_H gene arrangement was analyzed by nested RT-PCR.

Amplification of V_H and CDR3 Genes

V_H and CDR3 genes were amplified by PCR using 5' primers specific for each leader sequence of the V_H1 to V_H6 families (V_HL1: 5'-CCATGGACTGGACCTGGAGG-3', V_HL2: 5'-ATGGACATACTTTGT TCCAGC-3', V_HL3: 5'-CCATGGAGTTTGGGCTGAGC-3', V_HL4: 5'-ATGAAAC ACCTGTGGTT CTT-3', V_HL5: 5'-ATGGGGTCAACCGCCAT CCT-3', V_HL6: 5'-ATGTCTGTCTCCTT CCTCAT-3') or FR 3 (5'-ACACGG CTGTGTATT-3'), and 3' primers complementary to the germline J_H regions (J_H: 5'-CCCTGGACCAGTGGCAGAGGAGT-3', J_H1,2,4: 5'-ACTCACGTTTGAT(T/C) TCCA (G/C)CTTGGTTCC-3', J_H3: 5'-GTACTTACGTTT GATATCCACTTTGGTCC-3', J_H5: 5'-GCTTACGTTTAATCTCCAGTCGTGTCC-3'). PCR was performed in a final volume of 50 μl of reaction buffer [50 mM Tris-HCl, pH 9.0, at 25° C, 20 mM $(NH_4)_2SO_4$, 3.0 mM $MgCl_2$] containing 50 μmol of deoxyribonucleotide triphosphates, 2.5 U of recombinant *Taq* polymerase, and 50 pmol of each primer. Amplification consisted of an initial denaturation step of 5 minutes at 94° C, followed by 30 cycles under standard conditions (denaturation 1 minute at 94° C, annealing 1 minute at 52–56° C, extension 1 minute at 72° C), with a final extension step of 10 minutes at 72° C. Reamplification of 1-μl aliquots of the initial PCR product was performed using the above-described primers and PCR conditions. Aliquots of the final reaction were analyzed by electrophoresis on a 2% agarose gel (Sigma) containing ethidium bromide.

Sequencing and Cloning of I-V_H and CDR3 Genes

PCR products were purified and cloned into the pGEM-TA vector. After transformation of JM109 competent cells, clones found by plasmid DNA restriction analysis to contain an appropriately sized insert were selected. The double-stranded DNA templates from 5 to 10 colonies containing V_H or CDR3 gene inserts were sequenced both by the method of Sanger et al

(1977) and using a sequencer. These analyses were performed in all cases with a high density of PCR bands.

Assignment of Ig Gene Sequences

To analyze B-cell clonality, the VDJ region or the CDR3 was assigned by using the approach of Fais et al (1998), which requires a sequence of seven consecutive nucleotides of D_H segment containing no more than two nucleotide changes. Monoclonal and oligoclonal B-cell clonal expansion indicated that the identity of the CDR3 gene sequence(s), from one or two B-cell clones in CSF cells, should be at 60% to 80%. Polyclonal B-cell clonal expansion implies variability at the joining sites of CDR3 gene sequences. To analyze somatic hypermutation, nucleotide sequences were aligned with those in the GenBank current database of the IgBLAS–Analysis of Ig sequences and Nucleotide BLAST program (Natural Center for Biotechnology Information, New Haven, Connecticut). By comparing each sequence with the germline sequences, mutations were defined on the basis of nucleotide changes in the V_H segment. Two nucleotide exchanges in a single codon were scored as a single replacement mutation.

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References

Adams CWM (1983). The general pathology of multiple sclerosis: Morphological and chemical aspects of the brain. Baltimore: Williams and Wilkins.

Allen D, Cumano A, Dildrop R, Kocks C, Rajewsky K, Rajewsky N, Roes J, Sablitzky F, and Siekevitz M (1987). Timing, genetic requirements and functional consequences of somatic hypermutation during B-cell development. Immunol Rev 96:5–22.

Allen D, Simon T, Sablitzky F, Rajewsky K, and Cumano A (1988). Antibody engineering for the analysis of affinity maturation of an anti-hapten response. EMBO J 7:1995–2001.

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, and Lipman DJ (1997). Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Res 25:3389–3402.

Archelos JJ, Storch MK, and Hartung HP (2000). The role of B cells and autoantibodies in multiple sclerosis. Ann Neurol 47:694–706.

Avasarala JR, Cross AH, and Trotter JL (2001). Oligoclonal band number as a marker for prognosis in multiple sclerosis. Arch Neurol 58:2044–2045.

Bahler DW and Levy R (1992). Clonal evolution of a follicular lymphoma: Evidence for antigen selection. Proc Natl Acad Sci USA 89:6770–6774.

Baranzini SE, Jeong MC, Butunoi C, Murray RS, Bernard CCA, and Oksenberg JR (1999). B cell repertoire diversity and clonal expansion in multiple sclerosis brain lesions. J Immunol 163:5133–5144.

Bruck W, Porada P, Poser S, Rieckmann P, Hanefeld F, Kretzschmar HA, and Lassmann H (1995). Monocyte/macrophage differentiation in early multiple sclerosis lesions. Ann Neurol 38:788–796.

Colombo M, Dono M, Gazzola P, Roncella S, Valetto A, Chiorazzi N, Mancardi GL, and Ferrarini M (2000). Accumulation of clonally related B lymphocytes in the cerebrospinal fluid of multiple sclerosis patients. J Immunol 164:2782–2789.

Compston DA, Morgan BP, Campbell AK, Wilkins P, Cole G, Thomas ND, and Jasani B (1989). Immunocytochemical localization of the terminal complement complex in multiple sclerosis. Neuropathol Appl Neurobiol 15:307–316.

Correale J and de Los Milagros Bassani Molinas M (2002). Oligoclonal bands and antibody responses in multiple sclerosis. J Neurol 249:375–389.

Cross AH (2000). MS: The return of the B cell. Neurology 54:1214–1215.

Cross AH, Trotter JL, and Lyons JA (2001). B cells and antibodies in CNS demyelinating disease. J Neuroimmunol 112:1–14.

Fais F, Ghiotto F, Hashimoto S, Sellars B, Valetto A, Allen SL, Schulman P, Vinciguerra VP, Rai K, Rassenti LZ, Kipps TJ, Dighiero G, Schroeder HW Jr, Ferrarini M, Chiorazzi N (1998). Chronic lymphocytic leukemia B cells express restricted sets of mutated and unmutated antigen receptors. J Clin Invest 102:1515–1525.

Ferguson B, Matyszak MK, Esiri MM, and Perry VH (1997). Axonal damage in acute multiple sclerosis lesions. Brain 120 (Pt 3):393–399.

Ganter P, Prince C, and Esiri MM (1999). Spinal cord axonal loss in multiple sclerosis: A post-mortem study. Neuropathol Appl Neurobiol 25:459–467.

Genain CP, Cannella B, Hauser SL, and Raine CS (1999). Identification of autoantibodies associated with myelin damage in multiple sclerosis. Nat Med 5:170–175.

Gray D and Matzinger P (1991). T cell memory is short-lived in the absence of antigen. J Exp Med 174:969–974.

Gray D and Skarvall H (1988). B-cell memory is short-lived in the absence of antigen. Nature 336:70–73.

Griffiths GM, Berek C, Kaartinen M, and Milstein C (1984). Somatic mutation and the maturation of immune response to 2-phenyl oxazolone. Nature 312:271–275.

Hafler DA, Fox DA, Manning ME, Schlossman SF, Reinherz EL, and Weiner HL (1985). In vivo activated T lymphocytes in the peripheral blood and cerebrospinal fluid of patients with multiple sclerosis. N Engl J Med 312:1405–1411.

Hollowood K and Macartney JC (1991). Reduced apoptotic cell death in follicular lymphoma. J Pathol 163:337–342.

Jukes TH and King JL (1979). Evolutionary nucleotide replacements in DNA. Nature 281:605–606.

Liu YJ, Joshua DE, Williams GT, Smith CA, Gordon J, and MacLennan IC (1989). Mechanism of antigen-driven selection in germinal centres. Nature 342:929–931.

Lublin FD and Reingold SC (1996). Defining the clinical course of multiple sclerosis: Results of an international survey. National Multiple Sclerosis Society (USA) Advisory Committee on Clinical Trials of New Agents in Multiple Sclerosis. Neurology 46:907–911.

Lucchinetti C, Bruck W, Parisi J, Scheithauer B, Rodriguez M, and Lassmann H (2000). Heterogeneity of multiple sclerosis lesions: Implications for the pathogenesis of demyelination. Ann Neurol 47:707–717.

Macklin WB, Oberfield E, and Lees MB (1983). Electroblot analysis of rat myelin proteolipid protein and basic protein during development. Dev Neurosci 6:161–168.

Matsuda F, Ishii K, Bourvagnet P, Kuma K, Hayashida H, Miyata T, and Honjo T (1998). The complete nucleotide sequence of the human immunoglobulin heavy chain variable region locus. J Exp Med 188:2151–2162.

McDonald WI, Compston A, Edan G, Goodkin D, Hartung HP, Lublin FD, McFarland HF, Paty DW, Polman CH, Reingold SC, Sandberg-Wollheim M, Sibley W, Thompson A, van den Noort S, Weinshenker BY, Wolinsky JS (2001). Recommended diagnostic criteria for multiple sclerosis: Guidelines from the International Panel on the diagnosis of multiple sclerosis. Ann Neurol 50:121–127.

Noort S.v.d. and Holland NJ (1999). Multiple sclerosis in clinical practice. New York: DEMOS Medical Publishing.

Nossal GJ (1992). The molecular and cellular basis of affinity maturation in the antibody response. Cell 68:1–2.

Oste C (1988). Polymerase chain reaction. Biotechniques 6:162–167.

Owens GP, Kraus H, Burgoon MP, Smith-Jensen T, Devlin ME, and Gilden DH (1998). Restricted use of V(H)4 germline segments in an acute multiple sclerosis brain. Ann Neurol 43:236–243.

Poser CM, Paty DW, Scheinberg L, McDonald WI, Davis FA, Ebers GC, Johnson KP, Sibley WA, Silberberg DH, and Tourtellotte WW (1983). New diagnostic criteria for multiple sclerosis: Guidelines for research protocols. Ann Neurol 13:227–231.

Qin Y, Greiner A, Trunk MJ, Schmausser B, Ott MM, and Muller-Hermelink HK (1995). Somatic hypermutation in low-grade mucosa-associated lymphoid tissue-type B-cell lymphoma. Blood 86:3528–3534.

Qin YF, Duquette P, Zhang YP, Talbot P, Poole R, and Antel J (1998). Clonal expansion and somatic hypermutation of V-H genes of B cells from cerebrospinal fluid in multiple sclerosis. J Clin Invest 102:1045–1050.

Rissoan MC, Soumelis V, Kadowaki N, Grouard G, Briere F, de Waal Malefyt R, and Liu YJ (1999). Reciprocal control of T helper cell and dendritic cell differentiation. Science 283: 1183–1186.

Sanger F, Nicklen S, and Coulson AR (1977). DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467.

Shlomchik MJ, Aucoin AH, Pisetsky DS, and Weigert MG (1987). Structure and function of anti-DNA autoantibodies derived from a single autoimmune mouse. Proc Natl Acad Sci USA 84:9150–9154.

Siekevitz M, Kocks C, Rajewsky K, and Dildrop R (1987). Analysis of somatic mutation and class switching in naive and memory B cells generating adoptive primary and secondary responses. Cell 48:757–770.

Slifka MK, Antia R, Whitmire JK, and Ahmed R (1998). Humoral immunity due to long-lived plasma cells. Immunity 8:363–372.

Smith ME, Stone LA, Albert PS, Frank JA, Martin R, Armstrong M, Maloni H, McFarlin DE, and McFarland HF (1993). Clinical worsening in multiple sclerosis is associated with increased frequency and area of gadopentetate dimeglumine-enhancing magnetic resonance imaging lesions. Ann Neurol 33:480–489.

Tindall KR and Kunkel TA (1988). Fidelity of DNA synthesis by the Thermus aquaticus DNA polymerase. Biochemistry 27: 6008–6013.

Trapp BD, Peterson J, Ransohoff RM, Rudick R, Mork S, and Bo L (1998). Axonal transection in the lesions of multiple sclerosis. N Engl J Med 338:278–285.

Wucherpfennig KW, Catz I, Hausmann S, Strominger JL, Steinman L, and Warren KG (1997). Recognition of the immunodominant myelin basic protein peptide by autoantibodies and HLA-DR2-restricted T cell clones from multiple sclerosis patients: Identity of key contact residues in the B-cell and T-cell epitopes. J Clin Invest 100:1114–1122.