Published in final edited form as:

J Neuroimmunol. 2008 August 13; 199(1-2): 126–132. doi:10.1016/j.jneuroim.2008.04.031.

CSF IgG Heavy-Chain Bias in Patients at the Time of a Clinically Isolated Syndrome

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Abstract

Using FACS and single cell reverse transcriptase polymerase chain reaction, we examined the cerebrospinal fluid (CSF) IgG VH repertoires from 10 subjects with a clinically isolated demyelinating syndrome (CIS). B and plasma cell repertoires from individual subjects showed similar VH family germline usage, nearly identical levels of post-germinal center somatic hypermutation, and significant overlap in their clonal populations. Repertoires from 7 of 10 CIS subjects demonstrated a biased usage of VH4 and/or VH2 family gene segments in their plasma or B cell repertoires. V region bias, however, was not observed in the corresponding peripheral blood CD19+ B cell repertoires from 2 CIS subjects or in normal healthy adults. Clinically, subjects with VH4 or VH2 CSF IgG repertoire bias rapidly progressed to definite MS, whereas individuals without repertoire bias did not develop MS after a minimum of 2 years of follow-up ($p = 0.01$).

Keywords

B cells; neuroimmunology; multiple sclerosis; immunoglobulin; plasma cells

1. Introduction

Analysis of the humoral immune response in MS patients demonstrates features of an antigen-driven B cell and plasma cell response. Examination of the IgG heavy chain repertoire in MS plaques (Baranzini et al., 1999; Owens et al., 1998; Owens et al., 2001; Smith-Jensen et al., 2000) and CSF (Colombo et al., 2000; Owens et al., 2003; Qin et al., 1998; Ritchie et al., 2004; Harp et al., 2007) has revealed overrepresented and clonally related IgG sequences. Identical findings are observed in the CSF of individuals following a clinically-isolated demyelinating syndrome (CIS) [optic neuritis, brainstem or spinal cord event] (Haubold et al., 2004; Monson et al., 2005; Qin et al., 2003) indicating that similar forces shape the humoral immune response in early demyelinating disease.

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An additional feature of the central nervous system (CNS) IgG repertoire in MS patients is the overrepresentation of VH4 family heavy chain sequences in MS plaque, periplaque white matter, and CSF (Baranzini et al., 1999; Owens et al., 1998; Owens et al., 2007b; Smith-Jensen et al., 2000). The temporal course of VH4 bias in MS remains unknown. We therefore examined the VH family germline distribution in the CSF IgG B and plasma cell IgG repertoires of a cohort of CIS patients and examined the relationship between VH family germline bias and additional measures of disease activity.

2. Materials and methods

2.1. Patients

CSF was obtained from CIS patients (optic neuritis, brainstem or spinal cord syndrome) at the Neurosciences Center at the University of Colorado Hospital as a part of their standard clinical evaluation. Informed consent was obtained for study participation. Diagnoses included optic neuritis, transverse myelitis and brainstem syndrome. None of the patients received any immunomodulatory drugs or steroids within 1 month of CSF examination. Any subsequent diagnosis of definite MS was made using the revised McDonald criteria (McDonald et al., 2001; Polman et al., 2005) and was blinded to the results of the repertoire analysis.

2.2. Cell Labeling

CSF was centrifuged at 2000 rpm ($514 \times g$) for 10 min in an Eppendorf 5810R tabletop centrifuge. Under sterile conditions, the supernatant was removed and the cell pellet resuspended in a small amount of residual CSF. The cell suspension was incubated at room temperature with anti-human CD19-allophycocyanin, anti-human CD138-phycoerytherin and anti-human CD3-FITC (Caltag, Burlingame, CA), diluted with phosphate-buffered saline, and the tube was placed on ice.

2.3. Fluorescent-Activated Cell Sorting (FACS)

Individual CD138+CD3- plasma cells and CD19+CD3- B cells were sorted into 200 μ l wells of a 96-well PCR plate (ISC Bioexpress, Kayesville, UT) containing 20 μ l of cell lysis buffer using a MoFlo flow cytometer (Cytomations, Fort Collins, CO). CD19+ B cells that expressed CD138 were not excluded from the CD19+CD3- population. RNA from single cells in each well was reverse transcribed in the 96-well format and amplified in an I-cycler (Biorad) by nested PCR (Owens et al., 2003) using defined heavy and light chain leader, framework and constant region primers. If possible, two plates of B cells and plasma cells were collected from each sort. A single plate of B cells and a single plate of plasma cells were immediately reverse-transcribed and stored at -80°C ; the remaining plates were immediately frozen at -80°C and reverse-transcribed and PCR amplified at a later date. For study subjects ON03-1, ON03-3, ON03-5, CIS03-1, CIS04-3, ON04-7, and ON05-2, V regions from both plasma and B cells were amplified by nested RT-PCR. For subject ON04-8, B cell V regions were not amplified by nested RT-PCR. For subjects ON03-4 and ON04-10, V regions from CD138 plasma cells were not amplified despite multiple rounds of RT-PCR.

2.4. Screening and Purification of PCR Products

PCR products were screened by agarose gel electrophoresis for V-region sizes. Positive PCR products were purified from primers using the Montage PCR purification system (Millipore) according to the manufacturer's protocol. Sample concentrations were estimated by comparing fluorescence of the PCR product to that of mass-calibrated molecular weight standards on an ethidium bromide-stained 1.5% agarose gel.

2.5. Sequencing the PCR Products and Analysis

The purified PCR products were sequenced at the University of Colorado Cancer Center DNA Sequencing and Analysis Core. Sequencing primers have been described (Owens et al., 2003). Sequences were analyzed and edited with Chromas Sequence analysis software. The edited sequences were then aligned to the V Base database of antibody variable domain sequences using DNAPlot (<http://www.mrc-cpe.cam.ac.uk>). The most similar germline segment was identified based on the resulting alignment and the extent of sequence homology to that segment was determined.

2.6. Statistics

The V_H family distribution observed in adult peripheral blood CD19+ B cells parallels germline family prevalence (Brezinschek et al., 1995; Huang and Stollar, 1993). A CD138+ plasma cell or CD19+ B cell V_H repertoire was considered biased when the V_H family distribution deviated significantly from germline prevalence (Cook and Tomlinson, 1995). The probability of an observed CSF V_H family distribution was calculated by Chi-square goodness-of-fit using the functional germline prevalences published by Cook and Tomlinson (1995). The probability of an observed VH4 or VH2 population in a specific repertoire was calculated by binomial distribution. Two-way tables with measures of association (Fisher's exact test) were performed using Stata 10.

3. Results

3.1. CIS Patients: Clinical Data

Cerebrospinal fluid (CSF) was collected from 10 subjects (7 women and 3 men) with either monosymptomatic optic neuritis or a clinically isolated brainstem or spinal cord syndrome. The clinical diagnosis, MRI lesion load and CSF findings are shown in Table 1. Eight of the ten subjects had ON. The remaining two subjects had clinical features of either brainstem (CIS03-1) or spinal cord demyelination (CIS04-3). CNS inflammation, as determined by MRI or CSF markers, was present in 9 of 10 subjects. T2-weighted MRI lesions were frequently observed (mean=4.6; range 0–21); gadolinium-enhancing (Gad+) lesions were found in only two individuals. Nine of ten subjects had OCBs; seven with an elevated CSF IgG index.

3.2. CD19+ and CD138+ VH IgG Repertoires Demonstrate Clonal Expansion

Although most CSF samples demonstrated only a modest pleocytosis, B and plasma cells were observed in all CIS subjects by flow cytometry (Table 2). CD19+ B cells and CD138+ plasma cells comprised 3.2% (range: 0.28 – 7.52%) and 1.4% (range: 0.12 – 2.96%) of the CSF mononuclear cell population.

We analyzed the IgG and IgM variable region (V-region) sequences expressed by individual B and plasma cells by single cell RT-PCR. Despite repeated rounds of RT-PCR, both CD138 and CD19 repertoires were not obtained for all subjects. Nevertheless, a total of 16 different CD138 and CD19 IgG repertoires were generated. The nucleic acid sequence of the V-region of the heavy chain was used to identify the representative germline family, and the amino acid sequence of the CDR3 region was used as a unique identifier of clonal populations. As described in both MS and CIS cohorts (Haubold et al., 2004; Monson et al., 2005; Qin et al., 2003; Ritchie et al., 2004), expanded B and plasma cell clonal populations were detected in the CSF of every subject (Table 2). While IgM-expressing CD19 B cells were identified, clonal expansion was observed only in the IgG-expressing CD19 and CD138 populations. The fraction of cells in clonal populations was significantly higher in the plasma cell than in the corresponding B cell population (76.0% vs. 32.0%, $p=0.006$; paired t-test). As found in RRMS patients (Ritchie et al., 2004), the same clonal populations were seen infrequently in the B and

plasma cell populations (mean $\pm$ SD: 22.8% $\pm$ 22.2%; range 0 – 62.5%). The percentage of common VH region sequences in the B and plasma cell populations increased to 34.1%, however, when the comparison included the entire B and plasma cell repertoires. The percentage of common sequences increased further to 52.6% when only the unique plasma cell clones were tallied. The increased percentage of shared sequences within the CD138 clonal population trended towards, but did not meet clinical significance ($p=0.07$; paired t-test).

3.3. VH4 and VH2 Family Germline Bias in CIS CD138 and CD19 CSF Repertoires

The human immunoglobulin repertoire utilizes 51 functional germline genes that are divided into seven families based on sequence homology (Cook and Tomlinson, 1995). The largest germline family is VH3 with 22 gene segments, followed by VH1 and VH4 with 11 gene segments each. VH family usage in the naïve peripheral blood CD19 B cell repertoire closely approximates the frequency of the VH family gene segments within the germline (Brezinschek et al., 1995; Huang et al., 1992). The CSF CD138 plasma cell VH family distribution deviated from germline frequencies, however, in 7 of 8 CIS subjects (Table 3). As previously observed in MS CD138 plasma cell repertoires (Owens et al., 2007b), CD138 VH family germline bias was not driven by clonal expansion as only 2 of 8 VH family germline distributions differed when tabulated by total or unique sequences. For subject ON04-7, the discrepancy between the total and unique CD138 plasma cell distributions was due to the high percentage of sequences in clonal populations (85%) and the limited number of unique sequences available for analysis. For subject ON03-3, the difference was due to a single large VH2 clone that accounted for 20% of the total sequences analyzed. The CD19 B cell IgG VH family germline distribution deviated from expected germline frequencies in 4 of 8 individuals (Table 4). The CD19 B cell IgM repertoires were polyclonal, and as expected, the observed VH family germline distributions deviated from expected germline frequencies in only 1 of 8 subjects (ON04-10). Total and unique CD19 VH family germline distributions differed only for subject ON04-7 where two large VH2 clones dominated the IgG repertoire.

As observed in MS plaque (Owens et al., 1998) and CSF (Owens et al., 2007b), the bias in the B and plasma cell IgG VH family distributions were due to an overrepresentation of VH4 and sometimes VH2 family germline sequences (Tables 3 and 4). In 3 (ON03-1, ON03-4, and ON04-10) of 10 subjects, the V_H family distribution in the CSF IgG B or plasma cell repertoire did not vary statistically from family germline prevalence (Cook and Tomlinson, 1995). In the naïve peripheral blood B cell pool, approximately 20% and 5% of the population is comprised of VH4 and VH2 family sequences, respectively. In 7 of 10 CIS subjects, however, a significant increase was observed in the number of VH4 or VH2 family sequences in CSF. In most instances, VH4 family germline sequences were increased in isolation; however, in two subjects (ON03-5 and ON04-7), both VH4 and VH2 family germline sequences were increased. In subject ON03-3, VH2 family sequences were significantly increased in isolation. When compared to CIS subjects with unbiased repertoires, subjects with biased IgG repertoires had a significantly elevated mean percentage of VH4 family germline sequences (56.5% vs. 19.4%, $p = 0.04$); the mean percentage of VH2 germline sequences, however, was not significantly different (10.3% vs. 6.3%, $p = 0.43$).

As in healthy volunteers, the VH family distribution of CD19 B cells sorted from the peripheral blood of a subset of CIS subjects did not differ from germline prevalence (Table 5). A minor deviation from germline prevalence was noted in the peripheral blood repertoire from subject NC05-1 due to an increased number of VH3 family germline sequences ($p = 0.05$). A similar increase in VH3 germline sequences was observed in the CSF CD19 IgM repertoire of subject ON04-10 (Table 4). 84% of the IgM VH sequences in the CIS peripheral blood repertoires were nonmutated germline sequences (0 – 1 mutations) and probably represent naïve B cells.

3.4. VH4 or VH2 Family Bias Correlates With Conversion to Definite MS

Seven of ten CIS subjects reached a diagnosis of MS by the McDonald criteria within 6 months of their initial demyelinating event, six of the seven individuals reaching a diagnosis of clinically-definite MS (CDMS). The relationship between MRI risk, oligoclonal banding, VH4 or VH2 bias in the plasma or B cell repertoire and conversion to MS is shown in Table 6. The presence of VH4 or VH2 repertoire bias in either the plasma or B cell repertoire was strongly associated with conversion to McDonald MS ($p = 0.01$, Fisher's Exact Test) and CDMS ($p = 0.03$, Fisher's Exact Test). Nine CIS subjects were noted to have risk factors for conversion to McDonald MS or CDMS: T2-weighted MRI lesions on their initial MRI scan or OCBs in their CSF (Table 6). Repertoire bias, however, did not always correlate with a high-risk MRI (≥ 2 T2 lesions) or oligoclonal banding. The presence of repertoire bias was present in subjects (ON03-5, ON04-7, and ON05-2) with only a single or zero MRI lesions and was absent in some subjects (ON03-1 and ON04-10) with high risk MRIs and CSF OCBs.

4. Discussion

We used FACS and single cell RT-PCR to determine the IgG VH family distribution within the CSF B cell or plasma cell repertoire of CIS subjects. Most repertoires exhibited VH family distributions that differed from family germline prevalence. Overrepresented VH4 family germline sequences were found in 60% of the repertoires. One-third of the VH4-biased repertoires exhibited coincident VH2 family sequence bias, and one repertoire demonstrated isolated VH2 bias. Subjects with VH4 or VH2 repertoire bias converted to MS within the subsequent 6 months. In contrast, in our CIS subjects without repertoire bias, none developed MS within a minimum follow-up period of 2 years. The presence of VH4 or VH2 repertoire bias correlated with subsequent clinical activity, and in some subjects, was independent of T2-weighted MRI lesion load and OCBs. Two of three subjects with normal CSF IgG repertoires did not reach a diagnosis of McDonald MS or CDMS despite the presence of ≥ 2 T2-weighted MRI lesions or CSF OCBs at initial presentation. Three CIS subjects with VH4 or VH2 repertoire bias developed CDMS despite the presence of only a single T2-weighted brain lesion.

In the recent Betaferon in Newly Emerging Multiple Sclerosis for Initial Treatment (BENEFIT) trial (Kappos et al., 2006), CIS patients with at least two clinically silent T2-weighted brain MRI lesions were treated for two years with either placebo or interferon beta-1b. The probability of reaching either CDMS or definite MS by the McDonald criteria for placebo-treated patients was 20% and 51% at 6 months and 45% and 85% at 2 years, respectively. In our patient population, 70% of the subjects reached McDonald criteria MS within 6 months. Among our CIS subjects with VH4 or VH2 repertoire bias, 90% reached a diagnosis of CDMS, and 100% reached a diagnosis of McDonald MS. Presently, our study cohort is too small and the observation period is too short to know whether CSF VH4 or VH2 repertoire bias may be used to predict future disease activity or response to therapy.

CSF IgG B cell and plasma cell repertoires of CIS subjects demonstrate cardinal features of an antigen-driven humoral immune response: clonally-expanded, somatically hypermutated VH family sequences (Haubold et al., 2004; Monson et al., 2005; Qin et al., 2003). In addition, in a significant fraction of our CIS subjects, there was an overrepresentation of either VH4 or VH2 family germline segments in the CSF IgG B or plasma cell repertoire. A simple explanation is that the CDR regions of these germline families form a favorable conformation for binding the unknown antigenic target(s) in early MS. Such a phenomenon is observed during rotavirus (RV) infection, where B cells reactive against RV proteins 6 and 7 preferentially use VH1 and VH4 germline segments (Weitkamp et al., 2003). Interestingly, the two germlines that are overrepresented in CIS subjects, VH4 and VH2, are closely related based on nucleotide sequence homology in the framework 1 and framework 3 intervals (Kirkham et al., 1992). Since VH4 repertoire bias is evident in established demyelinating

disease (Owens et al., 2007b), the early presence of VH4 bias at the time of CIS may indicate that the target of the humoral immune response does not change significantly over time. This hypothesis, however, needs to be examined further by longitudinal analysis of MS B and plasma cell VH repertoires.

IgG repertoire bias has been found in several inflammatory disorders. Splenic germinal center B cells from patients with systemic lupus erythematosus (SLE) demonstrate a bias in VH5 gene family usage and an under-representation of VH1 family segments (Fraser et al., 2003). In patients with rheumatoid arthritis (RA), synovial tissue contains memory B cells with a high proportion of VH4 family segments (Voswinkel et al., 1996), although in individuals with ankylosing spondylitis (AS), VH4 family usage is under-represented while VH5 family usage is over-represented (Voswinkel et al., 2001). Like our CIS subjects with VH4 or VH2 CSF repertoire bias, VH repertoire bias observed in SLE, RA and AS patients may be a reflection of inflammatory activity or immunopathogenesis.

CSF IgG repertoire bias has not been extensively investigated in other inflammatory CNS disorders; however, VH segments recovered from subacute sclerosing panencephalitis CSF reveal an excess of VH1 rather than VH4 family segments (Owens et al., 2007a; Owens et al., 2007b). This suggests that compartmental VH4 or VH2 repertoire bias may not be a phenomenon of general CNS inflammation but have specific relevance to MS. Indeed, 3 of 5 CD19 B cell MS CSF repertoires reported by Harp and colleagues (2007) showed an overabundance of VH4 family sequences.

Although not yet practical clinically, CSF repertoire testing may be valuable to MS researchers evaluating predictors of subsequent disease activity in CIS cohorts. Further studies are needed to determine whether CSF VH4 or VH2 repertoire bias is an informative biomarker for MS diagnosis and prognosis.

Acknowledgements

This work was supported by Public Health Service grants NS32623 (J.L.B., D.H.G., G.P.O.), EY014573 (J.L.B.), NS041549 (M.P.B.) and the National Multiple Sclerosis Society Research Grant RG3908 (J.L.B.). K.H. was supported by a NIH Training Grant in Neurovirology-Molecular Biology (NS07321).

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Table 1

Clinical, MRI and CSF Features of CIS Patients

Subject No.	Age (years)	Sex	Clinical Data Clinical Presentation	Time to LP (mo)	Number Gad + MRI Lesions	Number T2 MRI Lesions	Cells	CSF IgG Index	OCBs
ON03-1	44	F	Optic Neuritis	10	0	7	5	2.0	6
CIS03-1	25	F	Myelopathy	2	0	5	0	1.4	6
ON03-3	32	M	Optic Neuritis	4	4	17	1	0.5	3
ON03-4	25	F	Optic Neuritis	1.5	0	0	2	0.5	0
ON03-5	23	F	Optic Neuritis	1.75	1	1	32	2.1	11
CIS04-3	44	M	Myelopathy	4	0	1 SC	4	0.93	9
ON04-7	27	F	Optic Neuritis	3	0	1 SC	6	1.17	7
ON04-8	49	F	Optic Neuritis	1.5	0	3	10	0.77	12
ON04-10	39	M	Optic Neuritis	1.25	0	3	3	0.51	0
ON05-2	35	F	Optic Neuritis	1	1	1	12	1.82	21

Abbreviations: LP, lumbar puncture; M, male; F, female; mo, months; Gad+, gadolinium-enhancing; T2, T2-weighted; SC, spinal cord lesions

Table 2

Characterization of CSF IgG Repertoires

Subject No.	Repertoire	% CSF Mononuclear Cells	No. Sequences Analyzed	% of sequences in clonal populations
ON03-1	CD19	7.14	27	30
	CD138	1.96	40	52
ON03-4	CD19	7.52	31	29
	CD138	2.96	N.A. ^B	N.A.
ON04-10	CD19	0.28	32	6
	CD138	0.12	N.A.	N.A.
CIS03-1 ^A	CD19	4.43	64	23
	CD138	0.68	78	77
ON04-8	CD19	0.92	N.A.	N.A.
	CD138	0.46	32	66
CIS04-3	CD19	1.67	N.A.	N.A.
	CD138	1.45	40	92
ON05-2	CD19	2.29	31	13
	CD138	1.29	28	82
ON03-5	CD19	4.78	48	50
	CD138	2.17	76	64
ON04-7	CD19	3.97	26	54
	CD138	0.98	61	85
ON03-3	CD19	2.53	69	54
	CD138	2.51	30	80

^A Repertoire analysis of CIS03-1 has been reported previously (Ritchie et al., 2004).

^B Not amplified - Despite multiple attempts at V region amplification, no heavy chain sequences were successfully recovered.

Table 3
VH family representation in the CSF CD138+ cells from CIS subjects^A

VHFamily	ON03-1	CIS03-1	ON03-3	ON03-5	CIS04-3	ON04-7	ON04-8	ON05-2
Total	n = 40	n = 78	n = 30	n = 76	n = 40	n = 60	n = 31	n = 28
VH1	17.5%	0.0%	26.7%	0.0%	0.0%	1.7%	19.4%	10.7%
VH2	7.5%	6.4%	20.0% *	15.8% #	0.0%	18.3% +	0.0%	14.3%
VH3	42.5%	51.3%	26.7%	17.1%	0.0%	43.3%	19.4%	10.7%
VH4	32.5%	42.3% +	26.7%	64.5% +	100% +	36.7% #	61.3% +	64.3% +
VH5	0.0%	0.0%	0.0%	2.6%	0.0%	0.0%	0.0%	0.0%
B χ^2	4.28	36.8	13.9	106.4	144.4	35.4	29.9	36.6
p ≤	0.368	0.0001	0.003	0.0001	0.0001	0.0001	0.0001	0.0001
Unique	n = 29	n = 31	n = 14	n = 44	n = 3	n = 18	n = 17	n = 12
VH1	20.7%	0.0%	28.6%	0.0%	0.0%	5.6%	11.8%	8.3%
VH2	10.3%	9.7%	7.1%	15.9% *	0.0%	16.7%	0.0%	8.3%
VH3	37.9%	32.3%	42.9%	15.9%	0.0%	44.4%	23.5%	16.7%
VH4	31.0%	58.1% +	21.4%	65.9% +	100% *	33.3%	64.7% +	66.7% #
VH5	0.0%	0.0%	0.0%	2.3%	0.0%	0.0%	0.0%	0.0%
B χ^2	3.51	28.7	0.91	65.1	10.8	7.55	18.7	14.9
p ≤	0.47	0.0001	0.92	0.0001	0.03	0.10	0.0001	0.005

^A Single CD138 + cells were sorted from CSF of 8 CIS subjects. IgG VH regions were amplified, sequenced and analyzed as described in Methods. Each column indicates the donor, number of functional VH sequences analyzed (n) and their percent distribution among different families calculated using the total and unique number of VH sequences analyzed. VH6 and VH7 germline segments were not detected in any repertoire. Numbers in boldface indicate a VH family whose abundance is significantly different from the expected germline prevalence by binomial probability.

^B A chi-squared goodness-of-fit compared the observed to the expected VH family distributions calculated from the germline prevalence published by Cook and Tomlinson (1995). After Bonferroni correction for multiple comparisons, $p \leq 0.006$ were considered statistically significant.

Germline Family Binomial Probability:

- * $p \leq 0.05$;
- # $p \leq 0.005$;
- + $p \leq 0.0001$.

VH family representation in CIS CSF CD19+ cells^A

VH Family	ON03-1 ^C		CIS03-1		ON03-3		ON03-4		ON03-5		CIS04-3 ^D		ON04-7		ON04-10		ON05-2	
	IgG n = 27	IgG n = 37	IgM n = 27	IgG n = 62	IgM n = 7	IgG n = 22	IgM n = 9	IgG n = 43	IgM n = 5	IgM n = 10	IgG n = 22	IgM n = 4	IgG n = 11	IgM n = 21	IgG n = 17	IgM n = 14		
Total	11.1%	0.0%	0.0%	29.0%	0.0%	18.2%	22.2%	2.3%	0.0%	20.0%	0.0%	20.0%	0.0%	9.1%	4.8%	11.8%	28.6%	
VH1	3.7%	8.1%	3.7%	4.8%	0.0%	9.1%	0.0%	9.3%	0.0%	10.0%	36.4% ⁺	0.0%	0.0%	4.8%	0.0%	11.8%	0.0%	
VH2	44.4%	56.8%	70.4% [*]	53.2%	57.1%	59.1%	33.3%	23.3%	80.0%	40.0%	40.9%	50.0%	81.8% [*]	90.5% ⁺	0.0%	57.1%		
VH3	37.0%	35.1% [*]	22.2%	12.9%	28.6%	13.6%	44.4%	62.8% ⁺	0.0%	30.0%	18.2%	50.0%	9.1%	0.0%	64.7% ⁺	7.1%		
VH4	3.7%	0.0%	3.7%	0.0%	14.3%	0.0%	0.0%	2.3%	20.0%	0.0%	4.5%	0.0%	0.0%	0.0%	0.0%	7.1%		
VH5	3.7%	14.5	10.7	7.8	4.33	3.28	3.28	46.5	7.34	1.04	39.5	2.80	6.47	19.0	21.0	3.51		
BX ²	0.32	0.005	0.03	0.05	0.22	0.51	0.51	<0.0001	0.11	0.90	<0.0001	0.59	0.16	<0.0001	<0.0005	0.47		
<i>p</i>																		
Unique	IgG n = 22	IgG n = 26	IgM n = 27	IgG n = 33	IgM n = 7	IgG n = 20	IgM n = 8	IgG n = 28	IgM n = 5	IgM n = 10	IgG n = 13	IgM n = 4	IgG n = 10	IgM n = 21	IgG n = 15	IgM n = 14		
VH1	13.6%	0.0%	0.0%	27.3%	0.0%	20.0%	25.0%	3.6%	0.0%	20.0%	0.0%	0.0%	10.0%	4.8%	13.3%	28.6%		
VH2	4.5%	11.5%	3.7%	6.1%	0.0%	10.0%	0.0%	14.3%	0.0%	10.0%	15.4%	0.0%	0.0%	4.8%	13.3%	0.0%		
VH3	50.0%	42.3%	70.4% [*]	48.5%	57.1%	55.0%	37.5%	25.0%	75.0%	40.0%	53.8%	50.0%	80.0%	90.5% ⁺	6.7%	57.1%		
VH4	27.3%	46.2% [#]	22.2%	18.2%	28.6%	15.0%	37.5%	53.6% ⁺	0.0%	30.0%	23.1%	50.0%	10.0%	0.0%	66.7% [#]	7.1%		
VH5	4.5%	0.0%	3.7%	0.0%	14.3%	0.0%	0.0%	3.6%	25.0%	0.0%	7.7%	0.0%	0.0%	0.0%	0.0%	7.1%		
X ²	1.30	15.4	10.7	2.2	4.33	2.42	1.84	23.1	7.46	1.04	5.61	2.80	5.36	19.0	21.3	3.51		
<i>p</i>	0.85	0.004	0.03	0.53	0.22	0.65	0.76	<0.0001	0.11	0.90	0.22	0.59	0.25	<0.0001	<0.0005	0.47		

^A Single CD19+ B lymphocytes were sorted from MS CSF and the respective IgM and IgG V regions were amplified and analyzed as described in Methods. Each column indicates the donor, total and unique number of functional V sequences analyzed (n) and their distribution among different VH families. Numbers in boldface indicate a VH family whose abundance is significantly different from the expected germline prevalence by binomial probability.

^B A chi-squared goodness-of-fit compared the observed to the expected VH family distributions based on germline prevalence reported by Cook and Tomlinson (1995). After Bonferroni correction for multiple comparisons, p ≤ 0.006 were considered statistically significant.

^C Only IgG V region sequences were amplified.

^D Only IgM V region sequences were amplified.

Germline Family Binomial Probability:

* p ≤ 0.05;

p ≤ 0.005;

+ p ≤ 0.0001.

Table 5
VH family representation of normal and CIS peripheral blood CD19 + B cells^A

VH Family	Germline n = 51	NC05-1 n = 50	NC05-2 n = 35	NC05-3 n = 63	ON03-5 n = 17	ON05-2 n = 37
VH1	21.6%	20%	17.1%	20.6%	11.8%	24.3%
VH2	5.9%	2%	0%	3.2%	6.1%	2.7%
VH3	43.1%	58%	57.1%	52.4%	70.6%	56.8%
VH4	21.6%	10%	25.7%	17.5%	17.6%	13.5%
VH5	3.9%	4%	0%	6.3%	0%	2.7%
VH7	2.0%	6%	0%	0%	0%	0%
χ^2, B	-	10.99	6.33	4.78	5.51	3.61
$p =$	-	0.05	0.28	0.44	0.23	0.46

^A Single CD19+ peripheral blood B cells were sorted from blood of normal healthy controls (NC) and CIS patients, and IgM VH regions were amplified, sequenced and analyzed as described in Methods. Most of the cells in each repertoire expressed non-mutated germline sequences and probably represented naïve B cells. Each column indicates the donor, number of functional VH sequences analyzed (n) and their distribution among different VH families. No VH6 germline segments were detected. Repertoire analyses of NC05-1, NC05-2, and NC05-3 have been reported previously (Owens et al., 2007b).

^B A chi-squared goodness-of-fit (5 degrees of freedom) compared the observed VH family distribution to the expected VH family distributions using the known germline prevalence of the 51 functional VH gene segments (Cook and Tomlinson, 1995).

Table 6

Subject	VH4-VH2 Repertoire Bias, MRI Lesion Load, Oligoclonal Banding and Conversion to Definite MS		High Risk MRI ^A		Oligoclonal Bands		VH4 Bias in CD138 Plasma Cells		VH2 Bias in CD138 Plasma Cells		VH4 Bias in CD19 B Cells		VH2 Bias in CD19 B Cells		MS Risk Based on VH4-VH2 Bias		Definite MS ^B		Time to Diagnosis	
ON03-3	+																			
ON03-5	-																			
ON04-7	-																			
ON04-8	+																			
ON05-2	-																			
CIS03-1	+																			
CIS04-3	+																			

ON03-1	+																			
ON03-4	-																			
ON04-10	+																			

^AHigh risk MRI is defined as 2 or more T2-weighted lesions white matter lesions on brain imaging

^BMS was diagnosed by either a new clinical event (Clinical) or a new MRI lesion (MRI). MRI diagnosis was made by the International Criteria (Polman et al., 2005).

^CN.A. - Not amplified

Abbreviations: mo, months