

Receptor Revision and Atypical Mutational Characteristics in Clonally Expanded B cells from
the Cerebrospinal Fluid of Recently Diagnosed Multiple Sclerosis Patients

Running Title: Analysis of CSF B cells from Multiple Sclerosis Patients

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ABSTRACT

Purpose: To determine whether cerebrospinal fluid (CSF) B cells exhibit clonal expansion in patients recently diagnosed with MS. CSF B cell clonal expansion was detected early in the disease process. Evidence of Receptor Revision was present in at least one MS patient who had been recently diagnosed with MS. Targeting of mutations to RGYW/WRCY motifs within CDRs was nominally observed in the CSF B cell clones, despite the high mutational frequencies. These observations are consistent with the presence of intense specific B cell stimulation and expansion in the CNS of MS patients early in the disease process.

Keywords: B lymphocytes, antibodies, autoimmunity, gene rearrangement

1. INTRODUCTION

Evidence that B cells may be involved in the development and perpetuation of MS (in at least a subset of patients)(reviewed in (Antel, 1999; Archelos et al., 2000; Cross et al., 2001; O'Connor et al., 2001)) include: 1] elevated immunoglobulin levels in the CSF of MS patients, 2] oligoclonal banding in the CSF of MS patients, 3] skewing of the $\kappa:\lambda$ ratio in the CSF of MS patients, 4] the presence of anti-myelin antibodies in the CSF of MS patients, 5] the demonstration that antibodies from the CSF of MS patients may contribute to the overall extent of tissue injury, and 6] the presence of plasma cells and antibody at lesion sites. In addition, MS patients with large numbers of B cells (and low monocyte numbers) in the CSF exhibit a faster rate of disease progression(Cepok et al., 2001). Furthermore, MS patients lacking CSF oligoclonal bands tend to have a more benign disease course(Stendahl-Brodin et al., 1979; Zeman et al., 2001). Taken together, these data strongly suggest that there is a specific B cell response in the CNS of MS patients that contributes to disease pathogenesis and tissue damage.

Local B cell clonal expansion in the CNS of MS patients provides evidence that B cells are responding to antigens in the CNS, and may be involved in at least one mechanism of MS pathogenesis. Indeed, data have been generated indicating that there is B cell clonal expansion by analysis of the immunoglobulin repertoire in spinal fluid B cells (Columbo et al., 2000; Owens et al., 2003; Qin et al., 2003; Qin et al., 1998) and B cells from brain lesions (Baranzini et al., 1999; Owens et al., 2001; Owens et al., 1998) of MS patients. A clone identified from a brain plaque in one of these cases was reactive against DNA (Williamson et al., 2001), which may be related to the initial pathogenesis of MS, but could also have been generated in response to the tissue damage occurring in the CNS of this patient with long-standing disease. If B cell

activation and clonal expansion were primary events in MS, they would be evident in early disease.

In order to investigate whether B cell clonal expansion could be detected in the CSF of patients with recent onset of MS, single cell PCR (SC-PCR) was utilized to characterize both the heavy and light chain repertoires from individual B cells obtained from the CSF of five MS patients, four of which had been recently diagnosed with MS as well as one patient who had been diagnosed with MS 15 years before this study. Analysis of the CSF B cell repertoires from all five MS patients supported the conclusion that there was specific local stimulation of B cell clonal expansion in the CNS of MS patients. In addition, since B cell clonal expansion was evident in all four patients recently diagnosed with MS, it becomes plausible that one of the primary events in the development of MS in at least a subset of MS patients is B cell activation. Whether these B cell clonal expansions play a relevant role in the pathogenesis of MS remains unknown at this time.

2. MATERIALS AND METHODS

Patient Description. See Table 1.

Cell preparation and cell sorting. Cerebrospinal fluid (CSF) cells were isolated by centrifugation of the CSF. CSF cells were stained with a PE-labeled anti-CD19 mAb. The CD19⁺ B cells from the CSF were sorted using a FACStar Plus flow cytometer (Becton Dickinson, San Jose, CA) outfitted with an automated cell deposition unit and one cell was deposited into each well of a 96-well PCR plate assembled on a microAmp base as described (Yavuz et al., 2002).

Primer extension preamplification, amplification of V_HDJ_H , $VκJκ$ and $VλJλ$ rearrangements and sequence analysis. These methods were carried out as previously described (Brezinschek et

al., 1995; Farner et al., 1999; Foster et al., 1997). Only productive rearrangements were analyzed as the limited number of nonproductive rearrangements did not allow for statistical analysis. Rearrangements that were clonally related were only counted once in the distribution studies. All sequences are available from the EMBL Data Bank (Accession Numbers Pending). Table 2 summarizes the number of B cells analyzed in each sampling, as well as the number of $V_{H/L}$ sequences recovered from each sampling.

Accuracy of the Sequencing Technique. The error rate of this sequencing method has been estimated to be approximately 1.0×10^{-4} per base pair (Monson et al., 2000).

Statistical Methods. Fisher's Exact Test was used to compare the distribution of heavy and light chain rearrangements found in the productive repertoires of the MS patients. P-values equal to or less than 0.05 were considered significant. Mutational frequencies (MF) were compared using the χ^2 test.

3. RESULTS

Differences in the V_H and V_L Repertoire in the PB and CSF

The distributions of V_H and V_L genes in the peripheral blood (PB) and CSF from the same patients were compared to determine whether the CSF B cell repertoire would be distinct from the peripheral blood B cell repertoire from the same patient. No significant deviations in individual $V_{H/L}$ gene distributions in the CSF of M354 or M484 compared to the PB of these same patients were noted (data not shown), although the V_H gene, 1-02 was overrepresented in M484 CSF in comparison to the same patient's PB.

Clonal Expansion is Evident in Patients Recently Diagnosed with MS

Two clonal populations from the CSF of M354 were identified (Table 3). Clone M354-A (n=4) from M354 CSF consisted of a $V_H3-48:J_H3$ heavy chain rearrangement and a $V_{\lambda}1G:J_{\lambda}7$

lambda light chain rearrangement. Clone M354-B from M354 CSF (n=3) also consisted of a V_H3-48:J_H3 heavy chain rearrangement, and was paired with a V_λ1C:J_λ1 light chain rearrangement. Of note, one example of this particular V_λ1C:J_λ1 rearrangement (including 8/10 of the same mutations, and the same CDR3 sequence composition) was identified in the PB of this same patient.

Three clonal populations were identified from the CSF of M484 (Table 3). Clone M484-A (n=2) consisted of a V_λ1G:J_λ7 light chain rearrangement, although the heavy chain was not recovered. The heavy chain for Clone M484-C (n=2) was also not recovered, but utilized a V_κB3:J_κ2 light chain rearrangement. Clone M484-B from M484 CSF (n=2) consisted of a V_H1-02:J_H4 heavy chain rearrangement and a V_κO2/O12:J_κ2 light chain rearrangement. Interestingly, the V_H1-02 rearrangement from Clone M484-B had extensive mutation accumulation (90.3% homology to germline), whereas its V_L pair, V_κO2/O12, did not contain any mutations. Such pairing (in which a heavily mutated V_H rearrangement is paired with a V_κ rearrangement with no mutations) occurs in only 26% of the normal donor peripheral B cell repertoire (Brezinschek et al., 1998). No clones identified in the CSF of this patient were identified in the PB, and none of the clones was similar to those found in the CSF of M354.

Three clonal populations were identified in the CSF of M875 (Table 3). Clone M875-A (n=2) consisted of a V_κA18:J_κ2 light chain rearrangement and Clone M875-C (n=2) consisted of a V_H3-66:J_H5 heavy chain rearrangement. The respective heavy and light chains were not recovered in these cases. Clone M875-B (n=2) consisted of a V_H3-09:J_H3 heavy chain rearrangement and a V_κA27:J_κ2 light chain rearrangement. Interestingly, just as had been found in clone M484-B, the V_H3-09 rearrangement from clone M875-B had extensive mutation

accumulation (93.9% homology to germline), whereas its V_L pair, $V\kappa A27$, did not contain any mutations.

M341, the patient with a different neurological disease, did not demonstrate any evidence of B cell clonal expansion in the CSF utilizing this single cell technique, even though this patient is known to have an anti-neuronal antibody titer (of unknown specificity). It is unclear why we were unable to detect an antigen driven B cell response in this patient.

Clonal expansion is also evident in a patient with well-established MS and demonstrated that light chain receptor revision likely occurred.

Seven clones were identified in the CSF of M368 (Table 4), the MS patient with well-established MS. All members of Clone M368-A (n=13) had a $V_H1-69:J_H6$ heavy chain rearrangement. Clone members M368-A1 (n=3) and A2 (n=2) rearranged the same $V\kappa$ gene (O2/O12), but their CDR3 sequences and distributions of mutations were discordant, and they had rearranged to different $J\kappa$ segments ($J\kappa2$ in the case of M368-A1, and $J\kappa1$ in the case of M368-A2). Light chain rearrangements for the remaining members of this clone were not amplified (Clone member M368-A3). Clone M368-B (n=5) also consisted of a $V_H1-69:J_H6$ heavy chain rearrangement, and was paired with a $V\kappa O2/O12:J\kappa1$ rearrangement three times (Clone member M368-B1), and a $V\kappa A27:J\kappa2$ rearrangement once (clone member M368-B2). Clone member M368-B3 also contained this $V_H1-69:J_H6$ rearrangement, but the productive V_L was not recovered. The heavy chain rearrangement from Clone M368-B was distinct from the heavy chain rearrangement identified in Clone M368-A. Heavy chain rearrangements for Clones M368-,D,E, F and G are as indicated in Table 4, although the light chains were not recovered.

Clonal expansion in a patient recently diagnosed with MS demonstrated that heavy chain receptor revision likely occurred.

Ten clonal populations were identified in the CSF of M125 (Table 5). Clone M125-A (n=7) had three separate clone members. All 7 clone members had the same V κ A27:J κ 2 light chain rearrangement, albeit with variations in mutational patterns, but not in CDR3 sequence. The heavy chain for Clone member M125-A1 (n=2) consisted of a V_H4-30.4:J_H6 heavy chain rearrangement, whereas the heavy chain for Clone member M125-A2 consisted of a V_H4-59:J_H6 heavy chain rearrangement. Heavy chain pairs for three of the clone members were not recovered. Clone M125-B (n=3) consisted of a V_H3-30:J_H6 heavy chain rearrangement paired with a V λ 3H:J λ 23 light chain rearrangement. Clone M125-C (n=2) had the same light chain rearrangement in both members (V λ 2A2:J λ 23), but was paired with a V_H3-07:J_H4 heavy chain rearrangement in one case and a V_H3-30:J_H4 heavy chain rearrangement in the other. Similarly, Clone M125-D (n=2) had the same light chain rearrangement in both members (V λ 2A3:J λ 1), but was paired with a V_H4-39:J_H2 heavy chain rearrangement in one case and a V_H3-64:J_H2 heavy chain rearrangement in the other. Clone M125-E (n=3) consisted of a V_H3-13:J_H6 heavy chain rearrangement paired with a V λ 1E:J λ 23 light chain rearrangement. Clone M125-F (n=2) consisted of a V_H1-46:J_H4 heavy chain rearrangement paired with a V κ O2/O12:J κ 4 light chain rearrangement. Clone M125-H (n=3) consisted of a V_H1-E:J_H5 heavy chain rearrangement paired with a V κ O2/O12:J κ 2 light chain rearrangement. Clones M125-G,I and J consisted of the light chain rearrangements as listed in Table 5, but the heavy chain pairs were not recovered.

Mutation Frequency, Intraclonal Diversity and Evidence of Light or Heavy Chain Replacement in CSF Clones

Clones from four of the five MS patients had elevated mutation accumulations, and some showed evidence of light or heavy chain replacement. For example, Clone M368-B utilized a V_H1-69 rearrangement that was heavily mutated (87.7% homology to germline), but was found paired with a $V_{\kappa}O2/O12:J_{\kappa}1$ rearrangement three times, and a $V_{\kappa}A27:J_{\kappa}2$ rearrangement once (Table 4). These light chains were also heavily mutated (percent homology to their germline genes: $V_{\kappa}O2/O12:J_{\kappa}1 = 93.5\% - 94.4\%$; $V_{\kappa}A27:J_{\kappa}2 = 93.2\%$). As proposed in Figure 1, there are at least three scenarios to account for the observed lineage of Clone M368-B.

All 13 members of Clone M368-A had a $V_H1-69:J_H6$ rearrangement that is distinct from that of Clone M368-B, but was also heavily mutated (91.3% – 93.2 % homology to germline V_H1-69 , Table 4). All of the $V_H1-69:J_H6$ rearrangements of Clone M368-A contained the same core of 13 mutations, with the same CDR3 sequence, although there is some variability in the position of additional mutations (Figure 2, Panel A). The light chain rearrangements identified in 5 of the 13 members of this clone were also heavily mutated (89.8% - 96.3% homology to germline $V_{\kappa}O2/O12$, Table 4 and Figure 2, Panel B). All 13 B cells from this clonal population also carried a nonproductive $V_{\kappa}B2:J_{\kappa}2$ rearrangement with minimal number of mutations (98.6% homology to germline) and the same CDR3 sequence. Resolving the relationship of Clone M368-A members cannot be ascertained, although it is clear that they are clonally related since all 13 clone members have the same V_HDJ_H rearrangement and nonproductive V_L rearrangement. However, since clone members M368-A1 and M368-A2 do not share the same light chain, it is likely that receptor revision had occurred in this clonal lineage as well.

Heavy chain replacement was observed in three of the clones from M125 (Clones A, C and D). All seven clone members of Clone M125-A had the same $V_{\kappa}A27:J_{\kappa}2$ light chain rearrangement, although 2 of them were paired with a $V_H4-30.4:J_H6$ heavy chain rearrangement,

and two others were paired with a V_H4-59:J_H6 heavy chain rearrangement. Interestingly, although the light chain rearrangement of this clone was somewhat mutated (95.0% - 100% homology to germline), the heavy chain pairs (both V_H4-30 and V_H4-59) were heavily mutated with germline homologies ranging from 88.8% to 93.8%. Both members of Clone M125-C had the same V_λ2A2:J_λ23 light chain rearrangement (95.2% - 96.1% homology to germline), but were paired with a V_H3-07:J_H6 heavy chain rearrangement in one case, and a V_H3-30:J_H4 heavy chain rearrangement in the other. Both heavy chain rearrangements had accumulated a large number of mutations with germline homologies ranging from 96.6% to 97.6%. Clone M125-D also utilized the same V_λ2A2:J_λ1 light chain rearrangement (97.4% - 98.7% homology to germline), but was paired with a V_H4-39:J_H2 heavy chain rearrangement in one case and a V_H3-64:J_H2 rearrangement in the other. The V_H4-39:J_H2 heavy chain rearrangement did not have any mutation accumulation, whereas the V_H3-64:J_H2 rearrangement had some accumulation of mutation (97.6% homology to germline).

Intraclonal diversity related to the accumulation of mutations within distinct members of a clone, such that some members of a clone have a particular number of mutations and other (presumably more recent) members will have accumulated additional mutations (Chen et al., 1997; Li et al., 2001; Prak and Weigert, 1995; Ruzickova et al., 2002). The clonal lineages described here demonstrated intraclonal diversity in most cases. For example, the light chain rearrangement from the earliest clone member of Clone M354-B (Figure 3A) had 98.2% homology to germline, and then accumulated 5 more mutations to generate the clone member with 96.1% homology to germline. The addition of 4 more mutations led to the generation of the clone member with 95.6% homology to germline. This schema does not rule out, however, that other clone intermediates existed, but that we did not detect them in this repertoire. The light

chain rearrangements from clone members M484-A, M368-B, M368-C, M125-A, -B, C, -D, -E, -G, -H and -J all demonstrate intraclonal diversity as illustrated in Figure 3. The heavy chain of Clone M368-A also exhibited intraclonal diversity (Figure 2A), as did the light chains of Clone M368 (Figure 2B). The heavy chain rearrangements from clone members M125-A, M368-D, -E, -F, and -G also demonstrated intraclonal diversity (Figures 4A and 4B). There were also clonal lineages in which we did not recover intermediate clone members, and thus did not demonstrate intraclonal diversity including the heavy chain rearrangements from Clone members M368-B, M368-C, M125-B through M125-F, and M125-H as well as the light chain rearrangements from Clone members M354-A, M484-C, M368-B2, M125-F, and M125-I (data not shown). Clones from M875 did not demonstrate intraclonal diversity within the heavy or light chain rearrangements.

Mutational Targeting to RGYW/WRCY motifs within the CDR's was not evident in the CSF repertoires.

Mutations are normally more frequent in the CDRs compared to the FRs of productive $V_{H/L}$ rearrangements, which is thought to reflect the focal role of CDR's in antigen binding and the necessity to restrict mutation accumulation in FRs in order to maintain structural integrity of the Ig (Dorner et al., 1998a). Analyses of the mutational characteristics from the CSF B cell clones (Table 6) indicated that some of the clones had mutational targeting into the CDR's. For example, the mutational frequency within the CDRs of Clone M354-B was significantly greater than the mutational frequency within the FRs of this clone. In contrast, the mutational frequency within the CDRs of Clone M354-A was no different than the mutational frequency within the FRs of this clone. In addition, previous analyses established the RGYW (purine, G, pyridine, A/T) motif on both DNA strands as a major target of the mutational machinery in both V_H and

V λ (but not V κ) gene rearrangements (Dorner et al., 1998b; Foster et al., 1999; Monson et al., 2000). Targeting of mutations to the CDRs of Clone M354-B did not extend to targeting of mutations into the RGYW motifs of the CDRs within this clone. No mutational targeting to the CDRs overall or in the context of RGYW motifs was observed in any of the three clones from M484. Mutational analysis of the clones from M368 was somewhat different in that the majority of the clones (both heavy and light chains) demonstrated mutational targeting to the CDRs in comparison to the FRs (see Clones A, B, C,D, and F). Notably, Clones M368-D and M368-F demonstrated mutational targeting to the CDRs in comparison to the FRs, as well as targeting of mutations within the RGYW motifs contained within the CDRs.

4. DISCUSSION

The primary goal of this study was to determine whether B cell clonal expansion could be detected in patients recently diagnosed with MS. Such a finding would support the idea that B cell clonal expansion is an early event in MS pathogenesis, and not simply a consequence of the disease process. Indeed, we have been able to identify clonal expansion in the CSF of four patients recently diagnosed with MS (one patient was diagnosed with Primary Progressive MS, and three with Relapsing Remitting MS). Detection of clones in such a small sample of CSF B cells is remarkable, and implies that they are highly represented in the inflammatory site, even as early as the time of diagnosis of MS. CSF B cell clonal expansion is not unique to MS patients since CSF B cells from patients with infectious disease of the CNS, such as neurosyphilis, Subacute Sclerosing Panencephalitis (SSPE), or viral meningitis also demonstrate B cell clonal expansion. However, analysis of these CSF B cell repertoires also indicated that extensive mutational accumulation was established in these patients within months of their diagnosis of

MS, and at least one of these recently diagnosed MS patients also had evidence of receptor editing.

Receptor editing/revision is a process resulting from persistent expression or re-expression of the recombination activating genes (RAGs) in B cells which re-initiate Ig gene recombination (Chen et al., 1997; Han et al., 1997; Kelsoe, 1996; Li et al., 2001; Papavasiliou et al., 1997; Prak and Weigert, 1995; Russell et al., 1991; Tiegs et al., 1993). The result is the deletion of the initial rearrangement and its replacement with a second rearrangement. This process is particularly obvious when V_k rearrangements are examined. The pairing of the same V_H1-69 gene rearrangement containing the same mutational pattern with two different light chains in Clone M368-B is suggestive that this clone underwent light chain replacement. Possible scenarios to account for the light chain replacement in this clone are shown in Figure 1. The factors that prompted clone member B1 to undergo light chain replacement cannot be ascertained. However, it is possible that this was an effort by the clone to neutralize its autoreactive potential. Similarly, Clone M368-A was also likely to have undergone light chain replacement, as proposed for Clone M368-B. Notably, however, Clone M368-A also had extensive mutation accumulation that was not observed in Clone M368-B. Since the clone was heavily mutated, it is likely that receptor revision began after mutational activity had been initiated. Therefore, this is likely to be an example of peripheral receptor revision resulting from the upregulation of RAG in germinal centers (Girschick et al., 2001).

Clones A, C and D from M125 also suggested that receptor revision had occurred, but at the heavy chain locus rather than the light chain locus. Heavy chain receptor editing is thought to occur for similar reasons as light chain editing/revision, but is oftentimes an unsuccessful process because of the mechanics of VDJ recombination (Nemazee and Weigert, 2000). In some

cases in which heavy chain receptor editing or revision has been documented, it occurred in parallel with somatic hypermutation (Wilson et al., 2000). It is therefore noteworthy that the clone with evidence of heavy chain receptor editing/revision had extensive mutation of both heavy and light chains. The finding that both Ig chains were extensively mutated suggests that receptor editing may have occurred during B cell ontogeny, possibly to avoid immunity to autoantigens expressed in the bone marrow during B cell development. However, this editing process may have eliminated the initial autoreactivity only to introduce reactivity to autoantigens expressed in other compartments, such as the central nervous system.

The CSF B cell clones expanded in these MS patients were likely to have been activated by an antigen driven process, as evidenced by their somatic mutation characteristics, (Tables 3-6). Interestingly, the V_H rearrangements from Clones M484-B and M875-B had extensive mutation accumulation, whereas their V_L pairs did not contain any mutations. Several scenarios could account for these particular results. For example, the antigen driving the clonal expansion of these particular pairings may preferentially bind to the unmutated light chain. Indeed, others have demonstrated that some light chains in germline configuration are able to bind myelin basic protein quite effectively (Galín et al., 1996). If this were the case, mutations of the light chain might cause a loss of antigen binding which would terminate expansion of these particular clones. Second, the mutations in these V_L rearrangements may have been removed in the process of peripheral light chain replacement. This would again suggest that RAG was upregulated in these clones as part of a germinal center reaction, or perhaps in “germinal center-like” reactions in the CNS (Harling-Berg et al., 1989; Hochwald et al., 1988; Knopf et al., 1995; Knopf et al., 1998; Phillips et al., 1997; Prineas, 1979; Sellebjerg et al., 2000; Torcia et al., 2001; Widner et al., 1988).

Notably, other processes that normally occur in germinal center reactions, such as targeting of mutations to RGYW/WRCY motifs in the CDRs, was not observed in the majority of CSF B cell clones (Table 6). In fact, RGYW/WRCY targeting within the CDRs was absent in all but two of the clones from the patient with well-established MS (M368-D and M368-F). None of the CSF B cell clones from the patients recently diagnosed with MS demonstrated RGYW/WRCY targeting within the CDRs. This was unexpected since mutations within the RGYW/WRCY motifs is a characteristic feature of germinal center reactions (Monson et al., 2001) and raises the question of whether CNS B cells have access to germinal centers or whether the CNS can itself provide a “germinal center-like” environment (Harling-Berg et al., 1989; Hochwald et al., 1988; Knopf et al., 1995; Knopf et al., 1998; Phillips et al., 1997; Prineas, 1979; Sellebjerg et al., 2000; Torcia et al., 2001; Widner et al., 1988), that is nonetheless defective in promoting RGYW/WRCY targeting. Another alternative could be that these CSF B cells do not require trafficking through a germinal center at all. In fact, it has been demonstrated by others that clonally expanded autoreactive B cells can undergo proliferation and somatic hypermutation outside of germinal centers (William et al., 2002).

It is compelling to conclude that the extent and complexity of CSF B cell clonal expansion will correlate with the duration of disease in most MS cases. However, the finding that M125, who had been diagnosed 4 months before sampling and had only 1 additional exacerbation following a diagnosis of optic neuritis also demonstrated clonal complexities and heavy chain replacement suggests that this may not always be the case, and is likely dependent on factors that we have yet to consider in our analysis, such as the possibility that the pathologic immune processes of MS likely begins before clinical symptoms emerge. This probability will make it particularly important to study the CSF B cell repertoires from patients who have not been

diagnosed with MS, but are considered at risk to do so. In any case, the existence of clonally expanded CSF B cells from all four of the patients with more recent onset of MS symptoms would suggest that these cells may contribute to the pathogenesis of the disease. Moreover, in all five MS patients analyzed, evidence of local antigen driven clonal expansion of B cells was detected in the CNS, strongly supporting the hypothesis that these cells play a pathogenic role in MS.

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Table 1: Patient Summary

	M368	M354	M484	M125	M875	M341
Type of MS	Relapsing Remitting ^a	Relapsing Remitting	Primary Progressive	Relapsing Remitting	Relapsing Remitting	NA ^f
Time since Diagnosis of MS	15 years	8 months	3 months	4 months	2 months	NA
Age/Sex	41/F	44/F	46/F	32/F	32/F	66/M
Initial Events	Transverse Myelitis	Transverse Myelitis	Progressive Myelopathy	Optic neuritis	Optic neuritis	Paraneoplastic syndrome
Exacerbation History	Multiple	2 additional	None	1 additional	None	NA
Treatment History Prior to LP	IV Steroids	None	None	IV Steroids	IV Steroids	NA
MRI findings	+	+	+	+	+	NA
Oligoclonal Banding (OCB)	Positive	Positive	Positive	Negative ^d	Positive	Positive
Ig Index ^b	2.57	0.8	3.12	0.6	ND ^e	ND
Ig Synthesis Rate ^c	34.6 mgs/24 hrs	8.3 mgs/24 hrs	50.4 mgs/24hrs	ND	4.2 mgs/24hrs	ND

^aM368 has recently advanced to secondary progressive MS

^bNormal Ig Index is 0.00 – 0,85

^cNormal Ig Synthesis Rate is 0.00 – 12.0 mgs/24 hrs

^dOCB present on a subsequent lumbar puncture

^e ND-Not determined

^fNA-Not applicable

Table 2: V_H/V_L Recovery

	M368	M354	M484	M125	M875	M341
Total number of CSF B cells analyzed	288	38	62	343	55	80
Total number of V _H sequences recovered	49	12	15	120	38	31
Total number of V _L sequences recovered	58	19	19	149	26	45

Table 3: Summary of Clones Identified in MS Patients M354, M484 and M875

		Number of Cells in Clone	Productive V _H Rearrangement	Homology Germline Gene	Productive V _L Rearrangement	Homology Germline Gene
M354	Clone A	4	3-48:X:J _H 3	95%	Vλ1G:Jλ7	96.9%
	Clone B ^a	3	3-48:X:J _H 3		Vλ1C:Jλ1	95.6% - 98.2%
M484	Clone A	2			Vλ1G:Jλ7	96.9% - 99.1%
	Clone B	2	1-02:X:J _H 4 ³	90.3%	VκO2/O12:Jκ2	
	Clone C	2			VκB3:Jκ2	
M875	Clone A	2			VκA18:Jκ2	100%
	Clone B	2	3-09:X:J _H 3	93.9%	VκA27:Jκ1	100%
	Clone C	2	3-66:X:J _H 5	99.3%		

^a This particular light chain rearrangement was also identified in the peripheral blood of this same patient.

Table 4: Summary of Clones Identified in MS Patient M368

		Number of Cells in Clone	Productive V _H Rearrangement	Homology Germline Gene	Productive V _L Rearrangement	Homology Germline Gene
M368	Clone A ^a					
	Member 1	3	1-69:X:J _H 6	93.2% - 92.7%	VκO2/O12:Jκ2	89.8% - 94.9%
	Member 2	2	1-69:X:J _H 6	92.7%	VκO2/O12:Jκ1	89.8% - 96.3%
	Member 3	8	1-69:X:J _H 6	91.3% - 93.2%		
	Clone B					
	Member 1	3	1-69:X:J _H 6	87.7%	VκO2/O12:Jκ1	93.5% - 94.4%
	Member 2	1	1-69:X:J _H 6	87.7%	VκA27:Jκ2	93.2%
	Member 3 ^b	1	1-69:X:J _H 6	87.7%		
	Clone C	3	3-30:X:J _H 4	92.4%	VκL12a:Jκ5	94.9% - 97.2%
	Clone D	2	3-11:X:J _H 5	91.7% - 92.2%		
	Clone E	2	1-02:X:J _H 4	86.8% - 93.1%		
	Clone F	2	1-03:X:J _H 5	94.1% - 93.6%		
	Clone G	3	DP-58:X:J _H 6	100% - 90.2%		

^a Each member of this clone had rearranged a nonproductive VκB2:Jκ2 (98.6% homologous to Germline Gene)

^b The productive light chain from one member of this clone was not recovered, but a nonproductive VκL2:Jκ5 rearrangement (93.6% homology to Germline Gene) was identified in this clone member

Table 5: Summary of Clones Identified in MS Patient M125

		Number of Cells in Clone	Productive V _H Rearrangement	Homology Germline Gene	Productive V _L Rearrangement	Homology Germline Gene
M125	Clone A					
	Member 1	2	4-30.4:X:J _H 6	93.8% - 91.4%	V _κ A27:J _κ 2	95.0%
	Member 2	2	4-59:X:J _H 6	88.8% - 89.1%	V _κ A27:J _κ 2	95.9% - 97.3%
	Member 3	3			V _κ A27:J _κ 2	97.3% - 100%
	Clone B	3	3-30:X:J _H 6	95.6%	V _λ 3H:J _λ 23	96.0% - 96.4%
	Clone C					
		1	3-07:X:J _H 4	96.6%	V _λ 2A2:J _λ 23	95.2%
		1	3-30:X:J _H 4	97.6%	V _λ 2A2:J _λ 23	96.1%
	Clone D					
		1	4-39:X:J _H 2	100%	V _λ 2A2:J _λ 1	98.7%
		1	3-64:X:J _H 2	97.6%	V _λ 2A2:J _λ 1	97.4%
	Clone E	3	3-13:X:J _H 6	93.6%	V _λ 1E:J _λ 23	96.1% - 97.0%
	Clone F	2	1-46:X:J _H 4	98.5%	V _κ O2/O12:J _κ 4	98.1%
	Clone G	2			V _κ L2:J _κ 1	95.4% - 96.8%
Clone H	3	1-E:X:J _H 5	93.8%	V _κ O2/O12:J _κ 2	98.2% - 100%	
Clone I	2			V _λ 2C:J _λ 23	99.1%	
Clone J	2			V _λ 2B2:J _λ 7	96.6% - 99.6%	

Table 6: Summary of Mutational Characteristics in Clones from the CSF of Three MS Patients

			Mutational Frequency in CDRs	Mutational Frequency in FRs	Percent Mutations in RGYW/WRCY's of CDRs	Percent Mutations in RGYW/WRCY's of FRs
M354	Clone A	Heavy	NA	NA	NA	NA
		Light	4.6	2.1	100	33.3
	Clone B	Heavy	NA	NA	NA	NA
		Light	4.6 /9.2 ^b	0 /1.4	50.0 /75.0 ^a	0 /50.0
M484	Clone A	Heavy	NA	NA	NA	NA
		Light	5.3	2.8	80.0	25.0
	Clone B	Heavy	12.1	8.6	50.0	50.0
		Light	0	0	0	0
	Clone C	Heavy	NA	NA	NA	NA
		Light	2.3/3.4	0/2.1	50.0/66.7	0/33.3
M368	Clone A	Heavy	15.2/16.7 ^b	5.2/7.8	70.0/100	0/30.0
		Light	6.7/17.3 ^b	2.1/9.9	57.1/80.0	20.0/42.9
	Clone B	Heavy	21.2 ^b	7.9	42.9	57.1
		Light-1	9.3/10.7 ^b	2.8/3.5	14.3/25.0	40.0/50.0
		Light-2	11.5 ^b	4.3	66.7	50.0
	Clone C	Heavy	21.2 ^b	3.9	57.1	14.3
		Light	8.3 ^b /13.0	1.1/5.0	14.3/16.7	42.9/50.0
	Clone D	Heavy	13.6 ^b	5.1	77.8 ^b	14.3
		Light	NA	NA	NA	NA
	Clone E	Heavy	9.1/15.2	3.6/10.9	30.0/50.0	20.0/26.7
		Light	NA	NA	NA	NA
	Clone F	Heavy	13.6/15.2 ^b	2.2	70.0/77.8 ^b	33.3
		Light	NA	NA	NA	NA
	Clone G	Heavy	10.6	9.4	100	38.5
Light		NA	NA	NA	NA	

^aminimum/maximum value^bsignificantly different from FR value

FIGURE LEGENDS

Figure 1: Lineage of Clone B. This clone was identified in the CSF of M368. The position of the V_{κ} genes of interest to this clone at the human V_{κ} loci are as indicated in the abbreviated physical map at the top of this figure. The dashed line segment indicates an 800 kb span of nucleotides between the proximal V_{κ} and distal V_{κ} cassettes, the latter of which rearranges by inversion (see text). Possible scenarios of how this clone evolved are outlined in the discussion.

Figure 2: Genealogical trees generated from clone M368-A heavy chain (Panel A) and light chain rearrangements (Panel B) demonstrating intraclonal diversity. The square at the bottom of each clone indicates the clone name and the $V_{H/L}$ rearrangement assigned to that clone. Each open square above the germline square represents one or more clone members with the same sequence, as well as the calculated percent homology to germline at that branch point. Mutations are indicated to the right of each branch, with the germline nucleotide listed first, followed by the nucleotide resulting from mutation, and the amino acid position(s) at which this mutation occurred. In some cases, gray squares represent an inferred intermediate.

Figure 3: Genealogical trees generated from the clonal light chain rearrangements of M354, M484, and M368 (Panel A), and M125 (Panel B) demonstrating intraclonal diversity. The square at the bottom of each clone indicates the clone name and the V_L rearrangement assigned to that clone. Each open square above the germline square represents one or more clone members with the same sequence, as well as the calculated percent homology to germline at that branch point. Mutations are indicated to the right of each branch, with the germline nucleotide listed first, followed by the nucleotide resulting

from mutation, and the amino acid position(s) at which this mutation occurred. In some cases, gray squares represent inferred intermediates.

Figure 4: Genealogical trees generated from the clonal heavy chain rearrangements of M368 (Panel A) and M125 (Panel B). The square at the bottom of each clone indicates the clone name and the V_H rearrangement assigned to that clone. Each open square above the germline square represents one or more clone members with the same sequence, as well as the calculated percent homology to germline at that branch point. Mutations are indicated to the right of each branch, with the germline nucleotide listed first, followed by the nucleotide resulting from mutation, and the amino acid position(s) at which this mutation occurred. Gray squares represent inferred intermediates.

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Figure 1: Lineage of Clone 10902-B

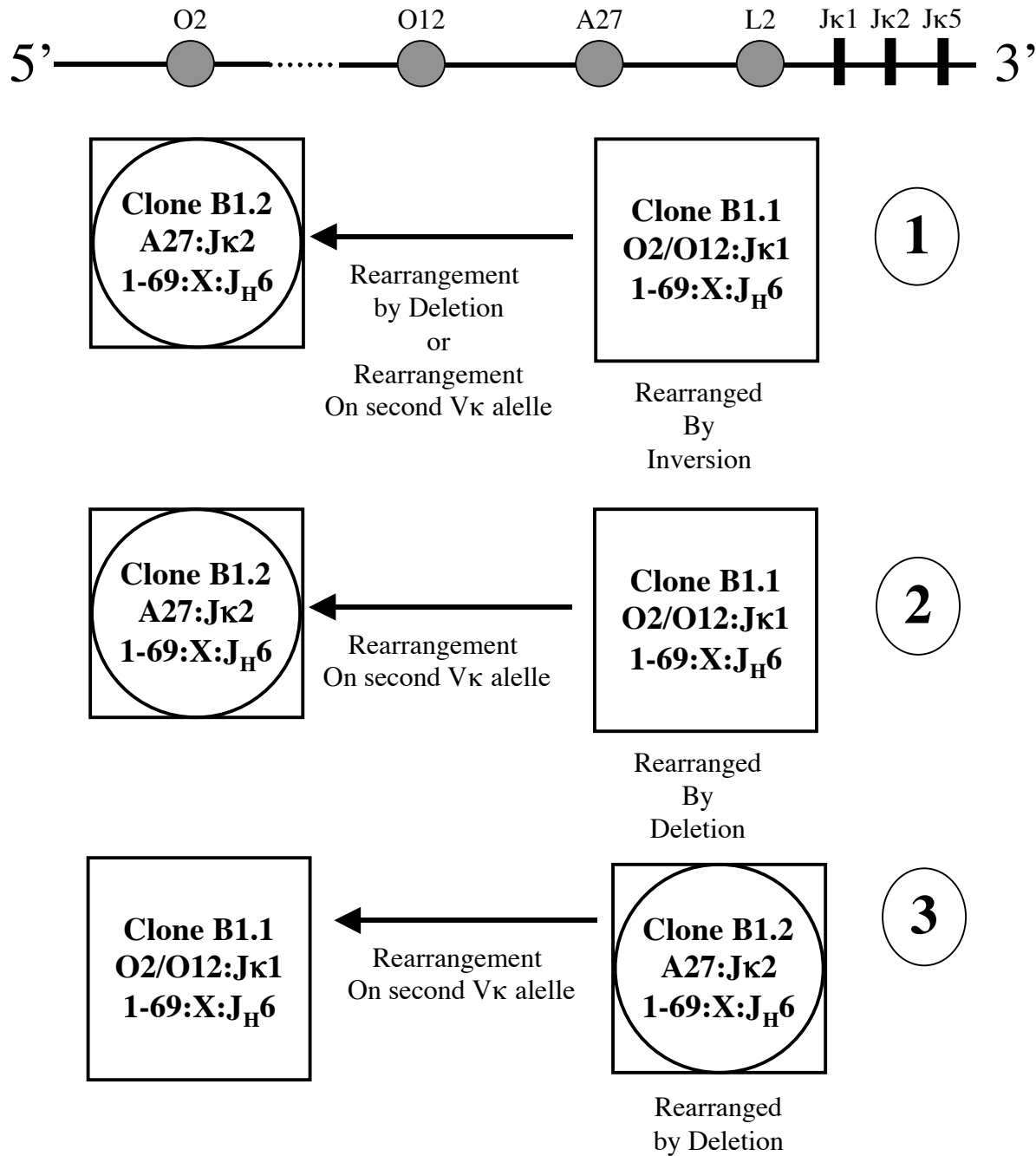


Figure 2A

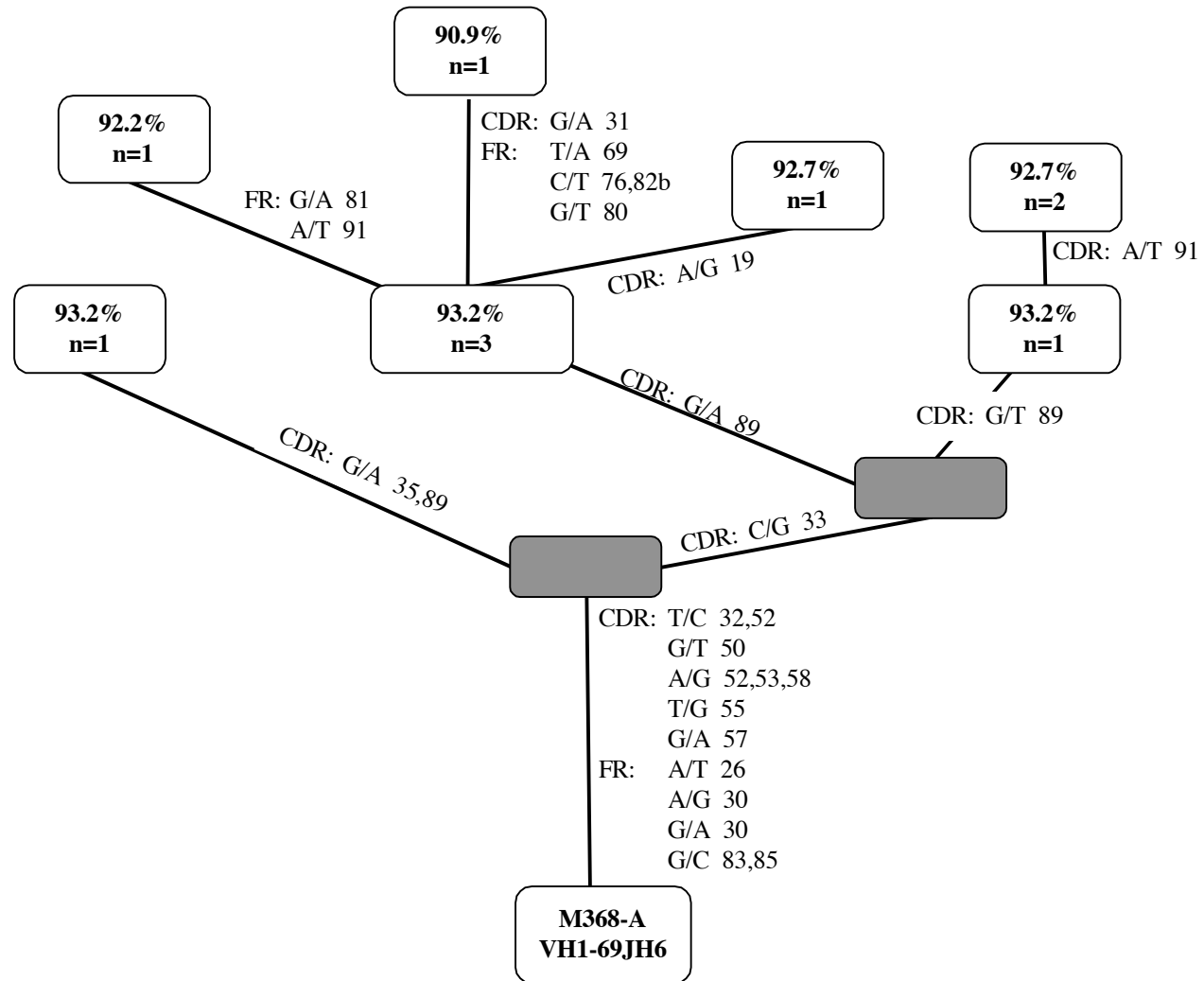


Figure 2B

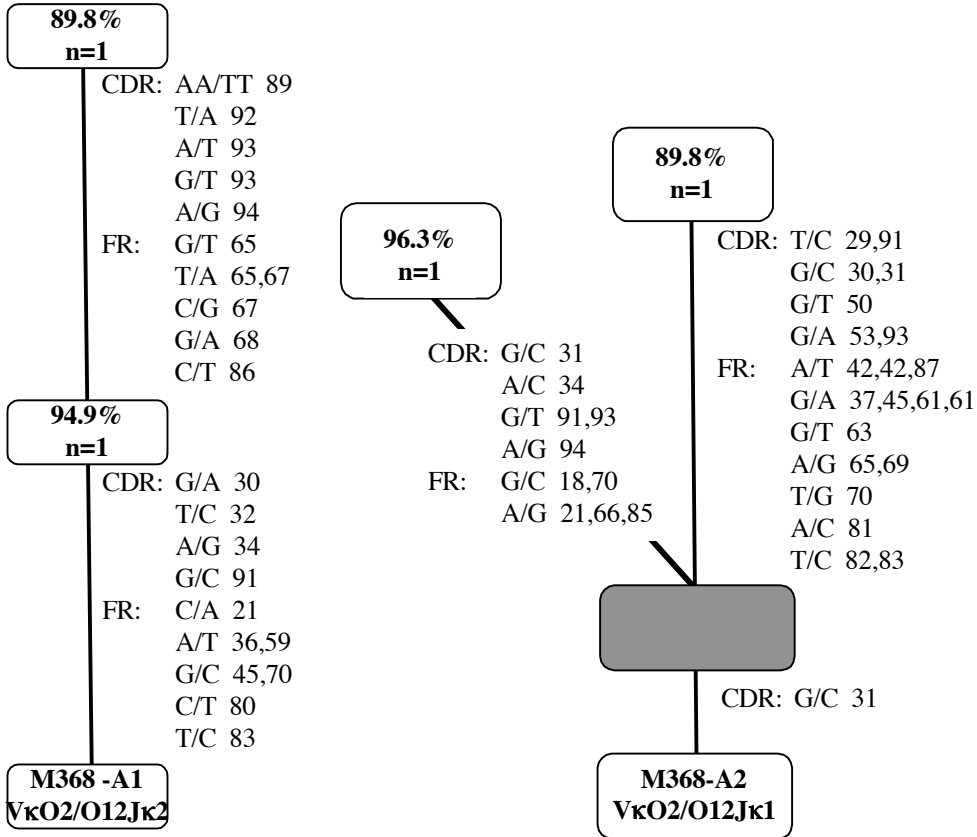


Figure 3A

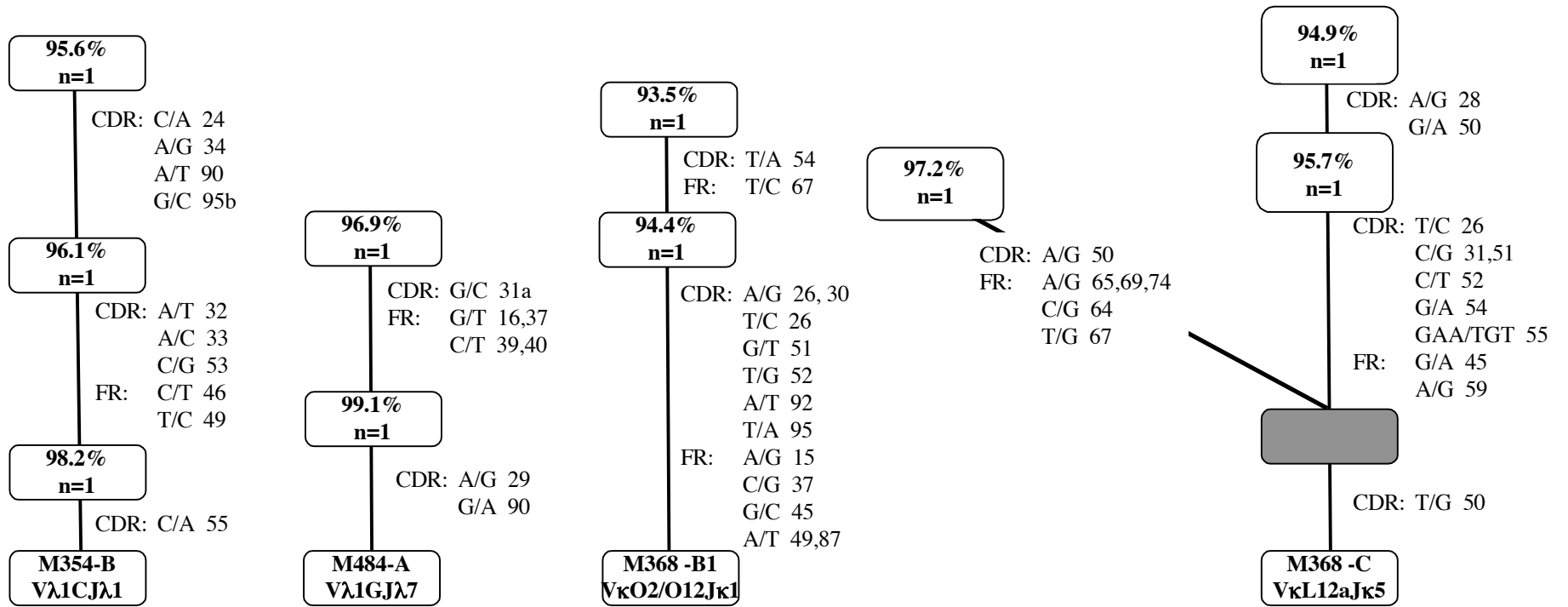


Figure 3B

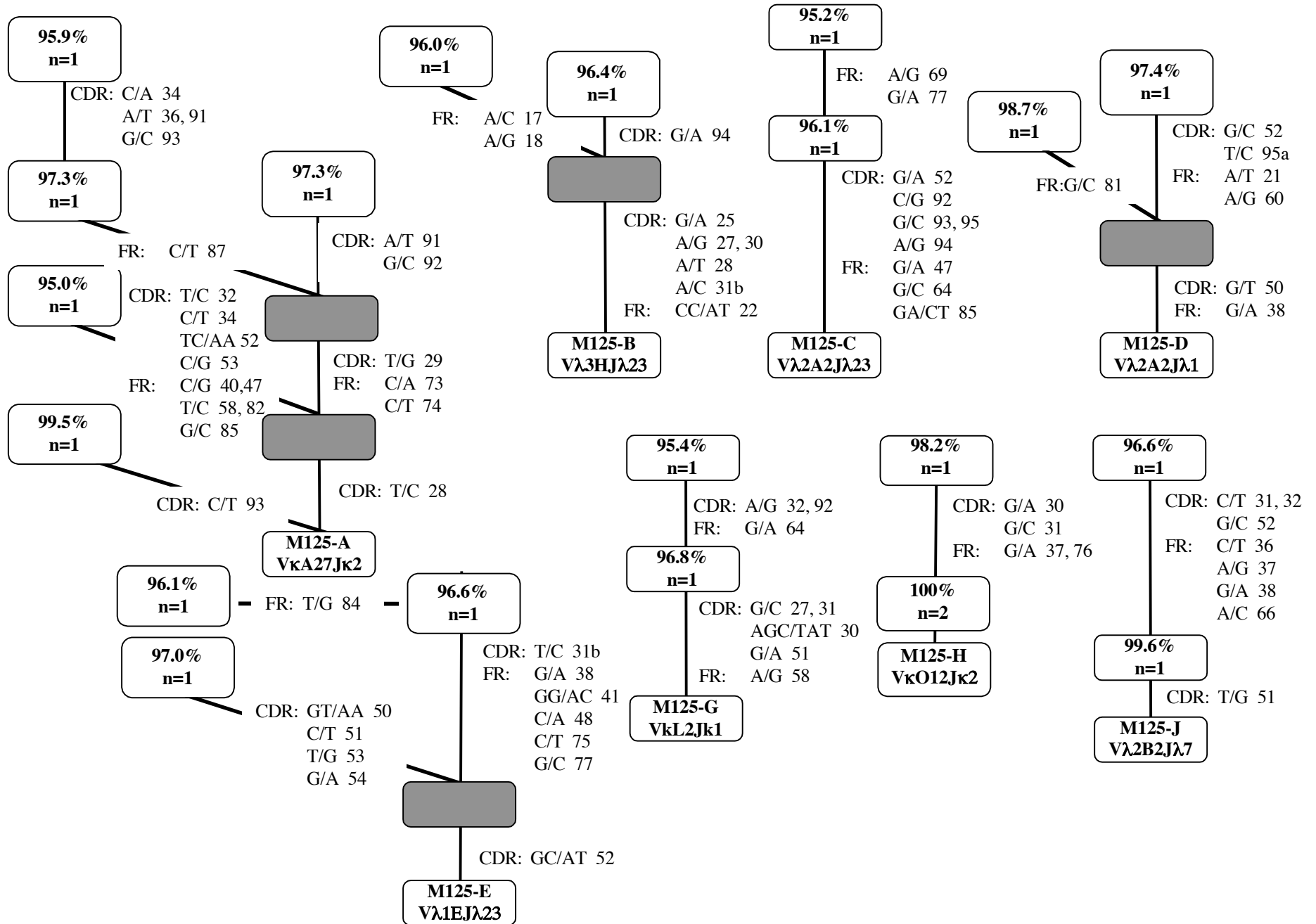


Figure 4A

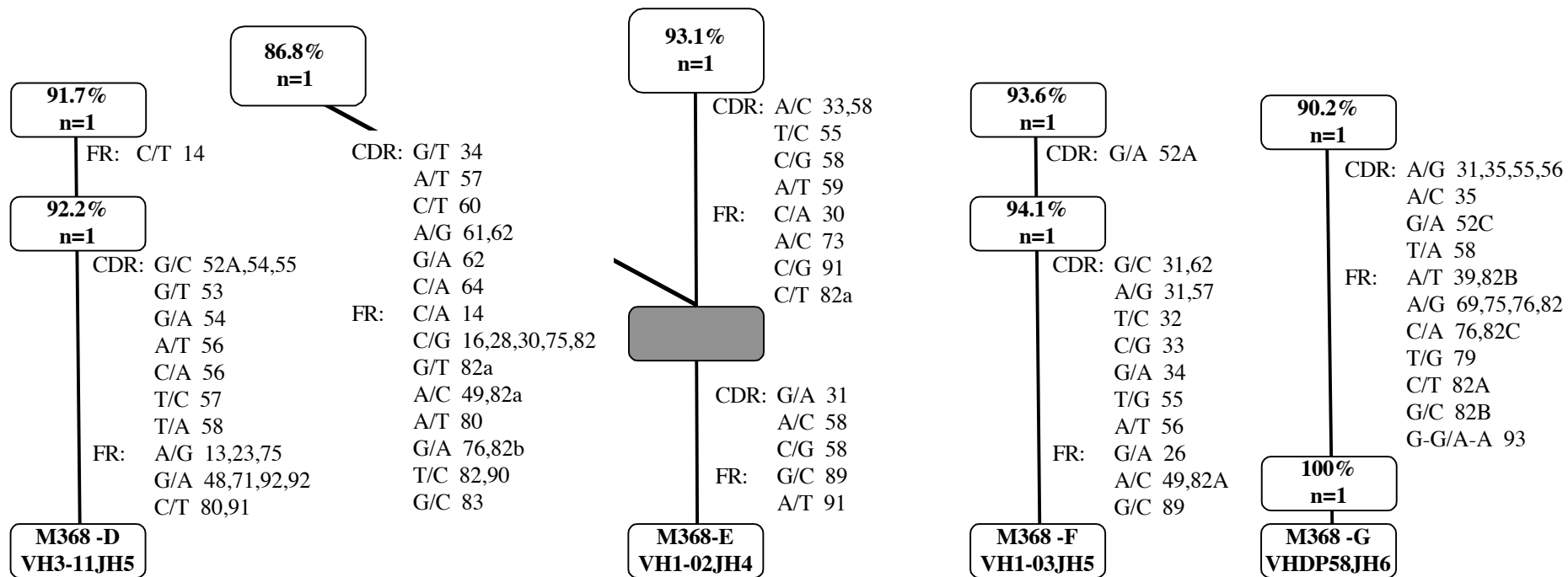


Figure 4B

