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Single-cell RT-PCR analysis of cerebrospinal fluid B cell clonality and immunoglobulin heavy and light chain variable region gene mutation in diffuse large B-cell lymphoma

Research Article

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Abbreviations: allophycocyanin, (APC); central nervous system, (CNS); cerebrospinal fluid, (CSF); complementarity-determining regions, (CDRs); Diffuse large B cell lymphoma, (DLBCL); dithiothreitol, (DTT); fluorescein, (FITC); fluorescence-activated cell sorting, (FACS); heavy chain variable, (VH); κ chain variable, (Vκ); magnetic resonance imaging, (MRI); phycoerythrin, (PE); Polymerase chain reaction, (PCR); reverse-transcription, (RT); variable, (V)

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Summary

Fluorescence-activated cell sorting and reverse transcription-polymerase chain reaction were used to examine cerebrospinal fluid and blood before and during treatment of a patient with diffuse large B cell lymphoma. A monoclonal population of IgM-expressing B cells that used a rearranged VH4 heavy chain variable region and Vκ3 light chain variable region was found. The persistent monoclonal B cell response in cerebrospinal fluid correlated with neurologic deterioration during treatment.

I. Introduction

Diffuse large B cell lymphoma (DLBCL) is a malignancy of mature B cells (Longo et al, 1993) containing immunoglobulin (Ig) gene rearrangements that express a homogeneous surface Ig receptor. The Ig binding sites are composed of 3 complementarity-determining regions (CDRs) separated by 4 relatively conserved framework regions (FRs). Ig mutations have been reported in DLBCL (Go et al, 2003), with prominent VH4 germline usage (Hsu and Levy, 1995; Shimizu et al, 2003). Polymerase chain reaction (PCR) has been shown to be a highly sensitive and rapid approach to identify clonally rearranged Ig genes in bone marrow and peripheral blood of patients with lymphoma and leukemia (Gleissner et al, 2002, Kienle et al, 2003). Herein, we report the application of fluorescence-activated cell sorting (FACS) and reverse-transcription (RT) PCR to analyze the rearranged variable (V) region sequence of single B cells in the cerebrospinal fluid (CSF) of a DLBCL patient with central nervous system (CNS) involvement.

II. Case report

In May 2003, a 36-year-old woman developed right occipital pain and peripheral facial weakness followed one month later by left peripheral facial weakness. In June 2003, a brain magnetic resonance imaging (MRI) scan was negative, but in July 2003, leptomeningeal enhancement was seen. The CSF contained increased numbers of mononuclear cells, all of which were malignant on cytologic examination. Both bone marrow and an axillary lymph node biopsy revealed stage IV large B cell lymphoma. She was treated intravenously with rituxan 500 mg/m^2, cyclophosphamide 750 mg/m^2, vincristine 1.4 mg/m^2, doxorubicin 50 mg/m^2, and oral prednisone 100 mg daily for 5 days. Treatment was repeated every 21 days for a total of 6 cycles. In August 2003, an Ommaya reservoir was inserted, and she received 16 intrathecal

treatments with methotrexate (12 mg) and Ara-C (70 mg). However, she developed persistent headache, seizures, mental status changes and left arm pain. In April 2004, MRI revealed progressive lymphomatous infiltration of brain white matter and the left brachial plexus. Despite whole-brain irradiation, she did not improve and was discharged to home hospice.

II. Materials and methods

A. Sample collection

CSF was analyzed before and during treatment, and blood was collected during treatment.

B. Fluorescence-activated cell sorting and cDNA synthesis

Blood was incubated with 1X ammonium chloride buffer, pH 7.4 (150 mM NH_4Cl, 10 mM $NaHCO_3$, 1 mM EDTA) to lyse red blood cells. CSF was centrifuged at 500 x g for 10 min at room temperature, and cells were suspended in 200 μl of residual fluid. White blood cells from blood and a CSF cell suspension were incubated for 30 min at room temperature with a mixture of monoclonal antibodies to human cell surface markers CD19 conjugated to allophycocyanin (APC), CD138 conjugated to R-phycoerythrin (PE), and CD3-conjugated to fluorescein (FITC) (CALTAG, Burlingame, CA). Sterile PBS was added to the labeled cells to a final volume of 700 μl, from which 500 μl was used for sorting with a MoFlo cytometer (Cytomations, Fort Collins, CO). Cells were first sorted by light scattering based on size and surface granularity, then sorted for $CD19^+CD3^-$ B cells and $CD138^+CD3^-$ plasma cells. Sorted cells were deposited individually into a 96-well PCR plate containing 20 μl of 1X RT reaction buffer (Invitrogen, Carlsbad, CA). Single cells were lysed in 24.5 μl of lysis buffer containing 1X RT buffer, 1.5 μg of random hexamers, 0.2 μM antisense constant region primer IgM C_H1 (Owens et al, 2003), and 0.9 % of Nonidet P-40 (Sigma, St. Louis, MO) at 65°C for 3 min, cooled to 25°C and maintained at 4°C. A mixture of 3 μl containing 0.1 M dithiothreitol (DTT), 0.5 μl RNase inhibitor (5 U), 1 μl 10 mM dNTPs, and 0.5 μl (l00 U) of Superscript II reverse transcriptase was added to each well to a final volume of 30 μl. RT was conducted at 37°C for 1 h, followed by 10-min incubation at 70°C to inactivate reverse transcriptase, and cooling to 4°C. Plates containing synthesized cDNA were stored at -70°C.

C. PCR amplification of heavy chain variable (VH) and κ chain variable (Vκ) genes

PCR primers used for IgM and IgG amplification are listed in **Table 2** of Owens et al, (2003) with modifications as described (Ritchie et al, 2004). Pooled family-based leader sequence primers for VH and Vκ families 1–5 were used in primary PCR with the conserved constant region primers IgG C_H1 and IgM CH1 for H chain, and Cκ1 for κ chain amplification. PCR amplifications were performed in a final 50-μl volume containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 0.01% gelatin, 100 μM dNTPs, 2 μM of primers, 2 U of *Taq* polymerase, and 5 μl of cDNA reaction mix. Cycling conditions included a 5-min denaturation at 94°C, followed by 34 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, ending with a 7-min extension time at 72°C. Nested PCR used 2 μl of the primary PCR reaction mix and a pool of family-based framework-1 primers in conjunction with a second, more 5'-conserved heavy chain constant region primer (IgM C_H2A or IgM C_H2) and k chain constant region primer Ck1D. Cycling conditions were the same as used in primary PCR. Except when specified, all PCR reagents were from Invitrogen.

D. Sequence analysis

PCR-amplified products were identified by 2% agarose gel electrophoresis and purified using the Qiaquick PCR purification kit (Qiagen, Valencia, CA). PCR products were sequenced directly and the sequences were analyzed with DNA PLOT (Centre for Protein Engineering, Cambridge, UK) and Immunoglobin BLAST (http://www.ncbi.nlm.nil.gov). The most homologous germline sequences were selected to calculate mutation ratios.

III. Results

B cell clonality was studied 3 times by FACS and RT-PCR: first in CSF on 7-11-03 before treatment; in blood on 11-03-03 during treatment while the patient was still symptomatic; and in CSF on 12-15-03, during continued treatment, while disease was worsening. On both occasions, cytologic examination revealed that 100% of all CSF mononuclear cells were malignant.

CSF and blood cells were sorted using a mixture of fluorescence-conjugated monoclonal antibodies to the human cell surface markers CD19, CD138 and CD3 (**Figure 1**). In the first CSF obtained before treatment, $CD19^+$ B cells constituted 17.8% of the selected cell population (**Figure 1B**), and no plasma cells were found (**Figure 1C**); in the second CSF obtained 6 weeks after treatment, $CD19^+$ B cells were found in 3.7% of selected cells. The blood contained almost no B cells during treatment one month later (**Table 1**).

The rearranged IgM H chain and κ L chain region sequences were amplified by RT-PCR from multiple B cells obtained before and during treatment. No IgG VH region transcripts were found. The first CSF PCR amplification efficiencies were 65% (62/96) for the IgM H chain (**Figure 2A**) and 83% (40/48) for the κ chain (**Figure 2B**).

Twenty randomly selected H and κ chain PCR products from each CSF analysis were purified and sequenced. A single monoclonal B cell population was present in CSF before and during treatment. **Figures 3 and 4** show the rearranged V sequences and the alignments of H and k chain variable regions, respectively, to their closest germline segments. Based on their CDR3 amino acid sequence and alignment to each other, all H and κ chain V-region sequences were identical. The VH region belongs to the VH4 family, with a CDR3 length of 14 amino acids and 85% homology to its closest germline DP-63. There were a total of 39 point mutations: 26 were within framework regions 1-3, and 13 were from CDR1 and CDR2. The replacement-to-silent mutation ratios (R:S) were 0.73 for the framework sequence, and 0.86 for CDR1 and CDR2 (**Table 2**). The L chain was κ-specific, belonged to the Vκ3 family, with CDR3 regions of 9 amino acids and 91% sequence homology to its closest germline DPK 22. There were 20 point mutations within the κ chain V region, and the R:S ratio was 1.86. The R:S ratios for framework and CDR were 1 and 7, respectively (**Table 2**). This same B cell clonal population was present in the second CSF during treatment and accounted for all

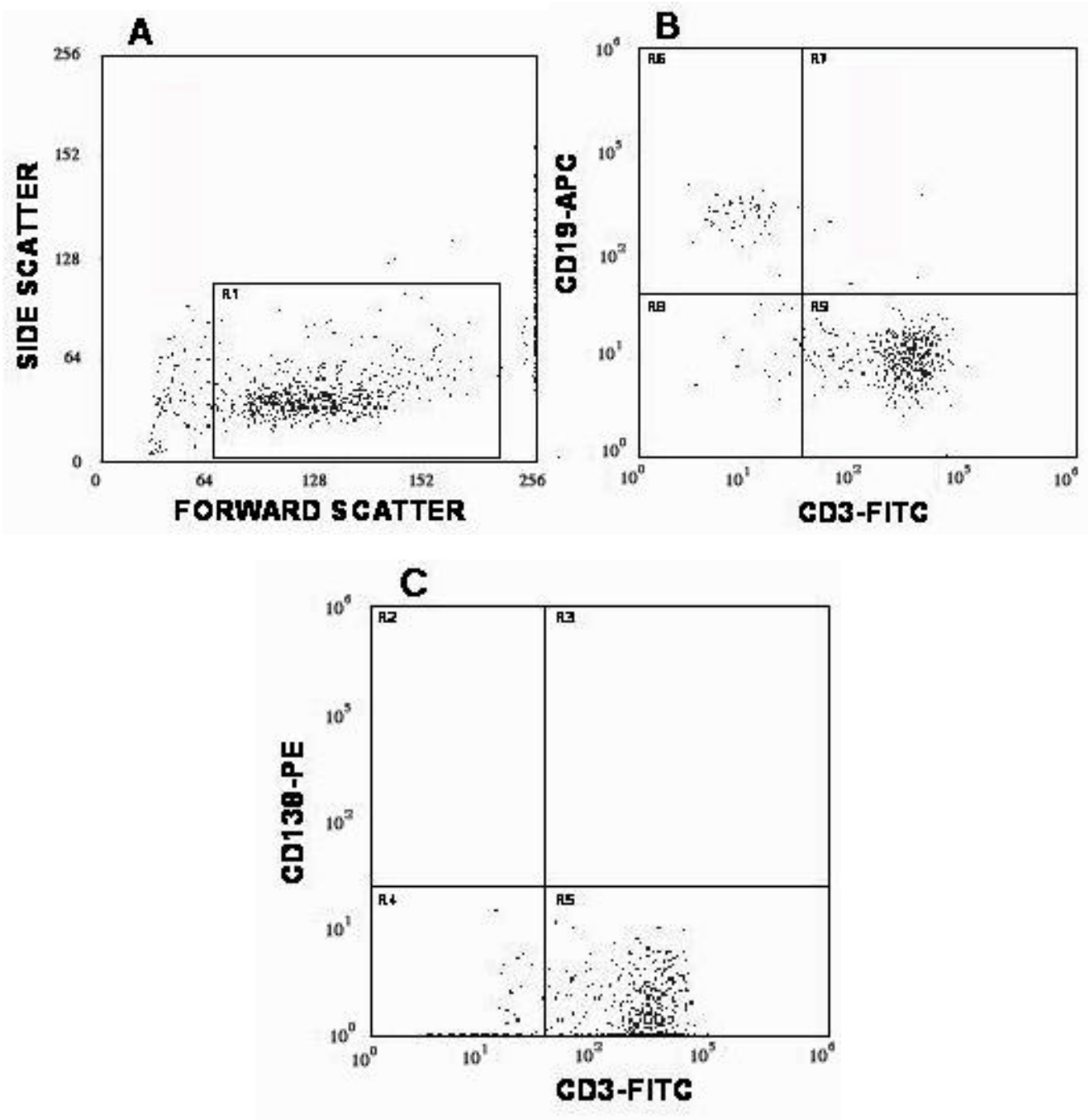

Figure 1. Fluorescence-activated cell sorting (FACS) of cerebrospinal fluid (CSF) B and plasma cells. CSF cells (7-11-03) were incubated with mouse monoclonal antibodies against human cell surface antigens CD3 (CD3-FITC), CD19 (CD19-APC) and CD138 (CD138-PE), as described in Materials and methods. The labeled cell mixture was sorted first by size (panel **A**, forward scatter) and surface granularity (side scatter). The size-selected cells (panel A, R1) were then separated into B cells (panel **B**, R6, $CD19^+CD3^-$) and plasma cells (panel **C**, R2, $CD138^+CD3^-$).

Table 1. B ($CD19^+CD3^-$) and plasma cell ($CD138^+CD3^-$) composition in CSF and blood

Sample	Date collected	Cell surface phenotype	Cell count	Abundance (%)
CSF	7/11/2003	$CD19^+CD3^-$	228	17.88
		$CD138^+CD3^-$	0	0
Blood*	11/3/2003	$CD19^+CD3^-$	2	0.15
		$CD138^+CD3^-$	1	0.07
CSF	12/15/03	$CD19^+CD3^-$	130	3.73

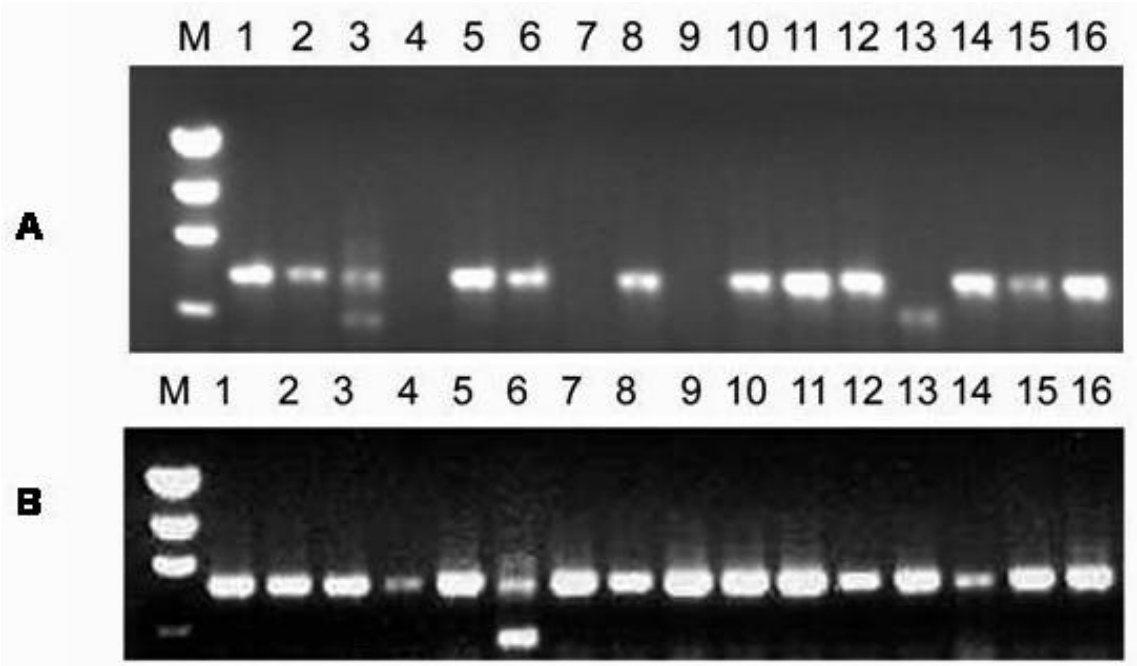

Figure 2. PCR amplification of the IgM variable region from single CSF B cells. Sorted cells from CSF (7-11-03) were lysed and cDNA was synthesized as described in Materials and methods. Pooled VH and Vκ leader primers and a constant region primer (IgM CH1 and IgM Cκ1) were used in primary PCR. Nested PCR was then performed with constant region primers CH2 and pooled VH framework primers, and constant region primers Cκ1D and pooled Vκ framework primers, respectively. Panel A shows an expected VH PCR product of ~450 bp, and panel B shows an expected Vκ PCR product of ~800 bp. M indicates molecular size markers, and numbers indicate the well number on the plate.

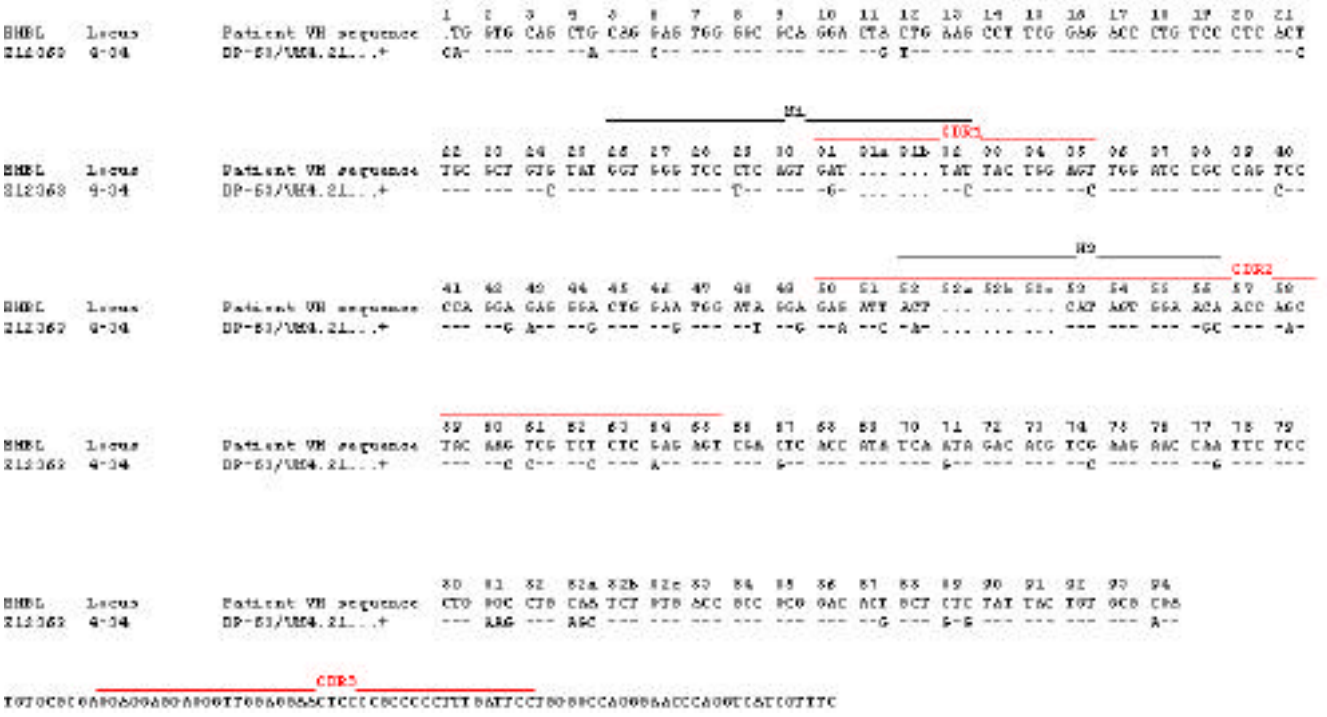

Figure 3. VH nucleotide sequence alignment with germline sequence. Representative VH sequence of CSF B cells were aligned with the most homologous VH germline gene in the database of DNA PLOT. The top line shows the subject's sequence, and the closest germline sequence (DP-63) is shown underneath, where capital letters indicate nucleotide differences, and dashes indicate identical sequences. H identifies the helix region. Based on alignment, there were a total of 39 point mutations.

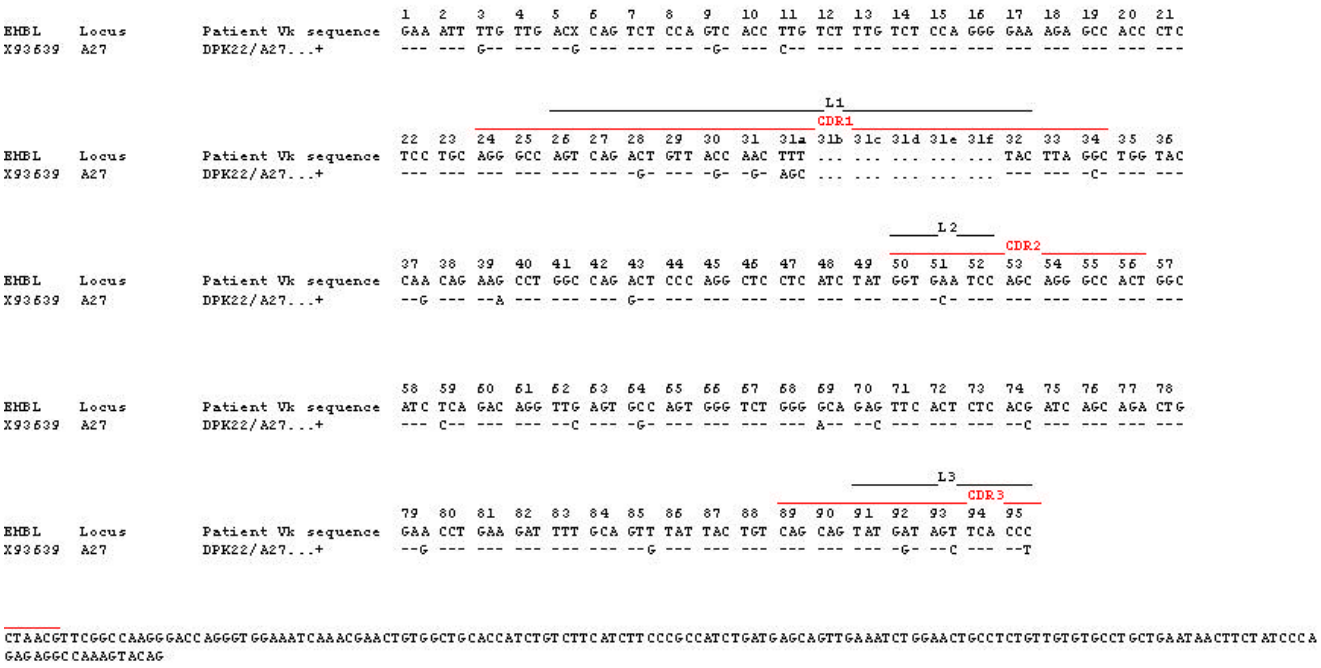

Figure 4. Vκ nucleotide sequence alignment with germline sequence. Representative Vκ sequence of CSF B cells were aligned with the most homologous Vκ germline gene in the database of DNA PLOT. The top line shows the subject's sequence, and the closest germline sequence (DPK22) is shown underneath, where capital letters indicate nucleotide differences, and dashes indicate identical sequences. L indicates the loop region. Based on alignment, there were a total of 20 point mutations.

Table 2. B cell IgM VH and Vκ gene rearrangements and mutations

V region	CDR3-IgM	Family	Germline	Homology (%)	R + S mutations (total)	R:S (total)	R:S (FR 1+2+3)	R:S (CDR 1+2)	JH	JL
VH	GGGGLEELPAPFDS	VH4-34	DP-63	85	39	17:22 (0.77)	11:15 (0.73)	6:7 (0.86)	JH4b	
Vκ	QQYDSSPLT	Vκ3	DPK22	91	20	13:7 (1.86)	6:6 (1)	7:1 (7)		Jκ1

The IgM VH and Vκ sequences were compared to the Vbase database. Their respective germline sequence homologies, number of mutations and replacement-to-silent (R:S) mutation ratios were determined.

B cells analyzed. Again, no IgG VH sequences were amplified from CSF.

IV. Discussion

Standard procedures to diagnose lymphoma include cytology, immunocytochemistry and flow cytometry. In addition, total DNA obtained from various tissues, including CSF, has been used to identify B cell monoclonality in lymphoma (Rhodes et al, 1996; Galoin et al, 1997; Storch-Hagenlocher et al, 2000; Hug et al, 2004). We show here that FACS and RT-PCR can be used to amplify Ig V region transcripts in single cells from human CSF, and that these techniques can identify B cell clonality in CSF in lymphoma with CNS involvement. Amplification and sequencing of IgM H and L chain (κ-restricted) V region genes confirmed B cell monoclonality in CSF; no IgG sequences were amplified in CSF. Sequence analysis revealed that the monoclonal B cells produced a functional Ig. The VH gene was most homologous with germline VH 4-34, consistent with previous findings in bone marrow of lymphoma patients (Huang et al, 1993; Shimizu et al, 2003).

Single-cell PCR analysis provides an exact pairing of VH and VL sequences that allows an accurate comparison of clonal populations. The PCR efficiency was as high as 83%, and B cell clonality was readily identified without subcloning. The monoclonality found in our patient's CSF differed from the polyclonal response found by FACS-RT-PCR when we analyzed single CSF B cells obtained on the first day of viral meningitis, before an antibody response would be expected (Owens et al, 2003). The persistent monoclonal B cell response in CSF of our patient correlated with her neurologic deterioration during treatment, much like the persistence of monoclonality produced by plasma cells in the bone marrow of patients with multiple myeloma (Szczepek et al, 1998).

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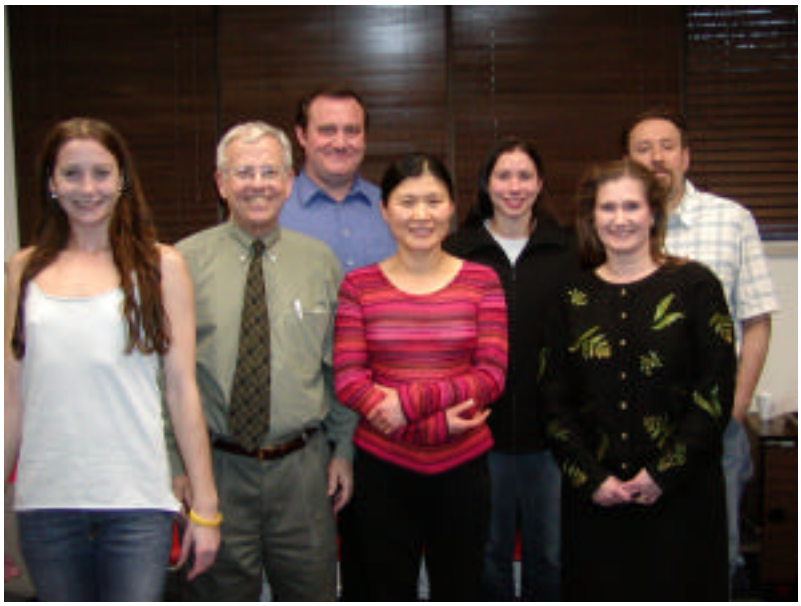

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