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

Xiaoli Yu[1], Gregory P. Owens[1], Alanna M. Ritchie[1], Mark P. Burgoon[1], Denise M. Damek[1], Kathryne M. Keays[1], and Donald H. Gilden[1,2]

[1] Department of Neurology

[2] Department of Microbiology, University of Colorado Health Sciences Center, Denver, Colorado, USA

*****Correspondence:** Xiaoli Yu, PhD, Department of Neurology, University of Colorado Health Sciences Center, 4200 E. 9th Avenue, Mail Stop B182, Denver, Colorado, 80262, USA. Telephone: 303-315-2393; fax: 303-315-8760; e-mail: xiaoli.yu@uchsc.edu

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 V 3 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$_4$Cl, 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$_H$1 (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 chain variable (V) 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 V families 1–5 were used in primary PCR with the conserved constant region primers IgG C$_H$1 and IgM CH1 for H chain, and C 1 for chain amplification. PCR amplifications were performed in a final 50-µl volume containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM MgCl$_2$, 0.01% gelatin, 100 µM dNTPs, 2 µM of primers, 2 U of *Taq* polymerase, and 5 µl of cDNA reaction mix. Cycling conditions included a 5-min denaturation at 94°C, followed by 34 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, ending with a 7-min extension time at 72°C. Nested PCR used 2 µl of the primary PCR reaction mix and a pool of family-based framework-1 primers in conjunction with a second, more 5'-conserved heavy chain constant region primer (IgM C$_H$2A or IgM C$_H$2) 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 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 chain (**Figure 2B**).

Twenty randomly selected H and 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 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 -specific, belonged to the V 3 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 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
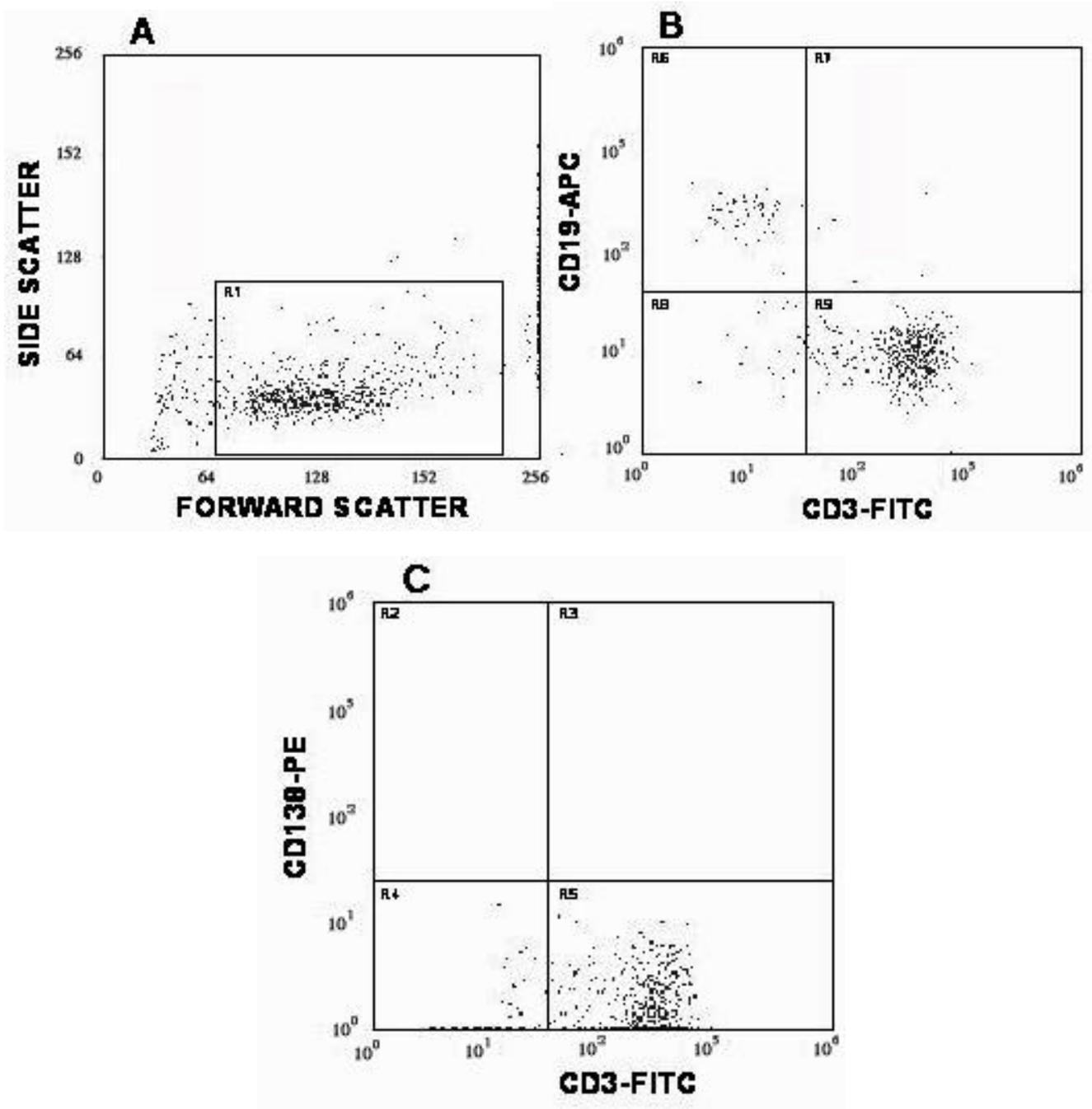
Figure 1. Fluorescence-activated cell sorting (FACS) of cerebrospinal fluid (CSF) B and plasma cells. CSF cells (7-11-03) were incubated with mouse monoclonal antibodies against human cell surface antigens CD3 (CD3-FITC), CD19 (CD19-APC) and CD138 (CD138-PE), as described in Materials and methods. The 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
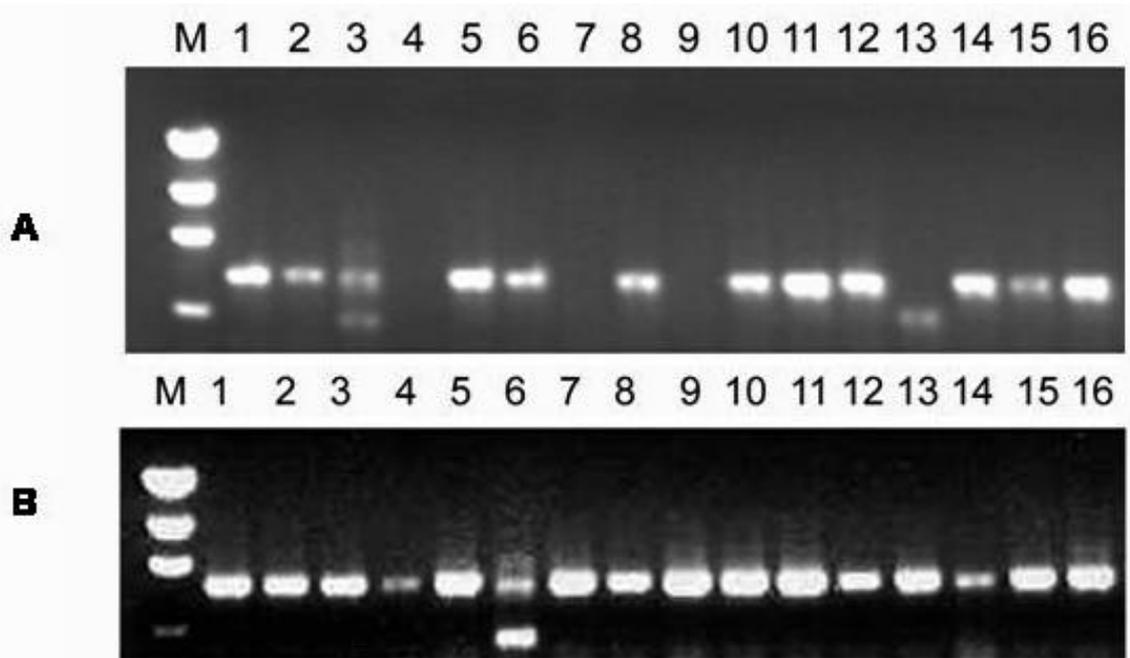
Figure 2. PCR amplification of the IgM variable region from single CSF B cells. Sorted cells from CSF (7-11-03) were lysed and cDNA was synthesized as described in Materials and methods. Pooled VH and V leader primers and a constant region primer (IgM CH1 and IgM C 1) were used in primary PCR. Nested PCR was then performed with constant region primers CH2 and pooled VH framework primers, and constant region primers C 1D and pooled V framework primers, respectively. Panel A shows an expected VH PCR product of ~450 bp, and panel B shows an expected V 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.

```
                                      1   2   3   4   5   6   7   8   9   10  11  12  13  14  15  16  17  18  19  20  21
EHBL    Locus   Patient Vk sequence   GAA ATT TTG TTG ACX CAG TCT CCA GTC ACC TTG TCT TTG TCT CCA GGG GAA AGA GCC ACC CTC
X93639  A27     DPK22/A27...+         --- --- G-- --- --G --- --- --- -G- --- C-- --- --- --- --- --- --- --- --- --- ---

                                                                                      L1
                                                                                      CDR1
                                      22  23  24  25  26  27  28  29  30  31  31a 31b 31c 31d 31e 31f 32  33  34  35  36
EHBL    Locus   Patient Vk sequence   TCC TGC AGG GCC AGT CAG ACT GTT ACC AAC TTT ... ... ... ... ... TAC TTA GGC TGG TAC
X93639  A27     DPK22/A27...+         --- --- --- --- --- --- -G- --- -G- -G- AGC ... ... ... ... ... --- --- -C- --- ---

                                                                              L2
                                                                              CDR2
                                      37  38  39  40  41  42  43  44  45  46  47  48  49  50  51  52  53  54  55  56  57
EHBL    Locus   Patient Vk sequence   CAA CAG AAG CCT GGC CAG ACT CCC AGG CTC CTC ATC TAT GGT GAA TCC AGC AGG GCC ACT GGC
X93639  A27     DPK22/A27...+         --G --- --A --- --- --- G-- --- --- --- --- --- --- -C- --- --- --- --- --- --- ---

                                      58  59  60  61  62  63  64  65  66  67  68  69  70  71  72  73  74  75  76  77  78
EHBL    Locus   Patient Vk sequence   ATC TCA GAC AGG TTG AGT GCC AGT GGG TCT GGG GCA GAG TTC ACT CTC ACG ATC AGC AGA CTG
X93639  A27     DPK22/A27...+         --- C-- --- --- --C --- -G- --- --- --- A-- --C --- --- --C --- --- --- ---

                                                                              L3
                                                                              CDR3
                                      79  80  81  82  83  84  85  86  87  88  89  90  91  92  93  94  95
EHBL    Locus   Patient Vk sequence   GAA CCT GAA GAT TTT GCA GTT TAT TAC TGT CAG CAG TAT GAT AGT TCA CCC
X93639  A27     DPK22/A27...+         --G --- --- --- --- --- --G --- --- --- --- --- --- -G- --C --- --T
```

```
CTAACGTTCGGCCAAGGGACCAGGGTGGAAATCAAACGAACTGTGGCTGCACCATCTGTCTTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAACTGCCTCTGTTGTGTGCCTGCTGAATAACTTCTATCCCA
GAGAGGCCAAAGTACAG
```
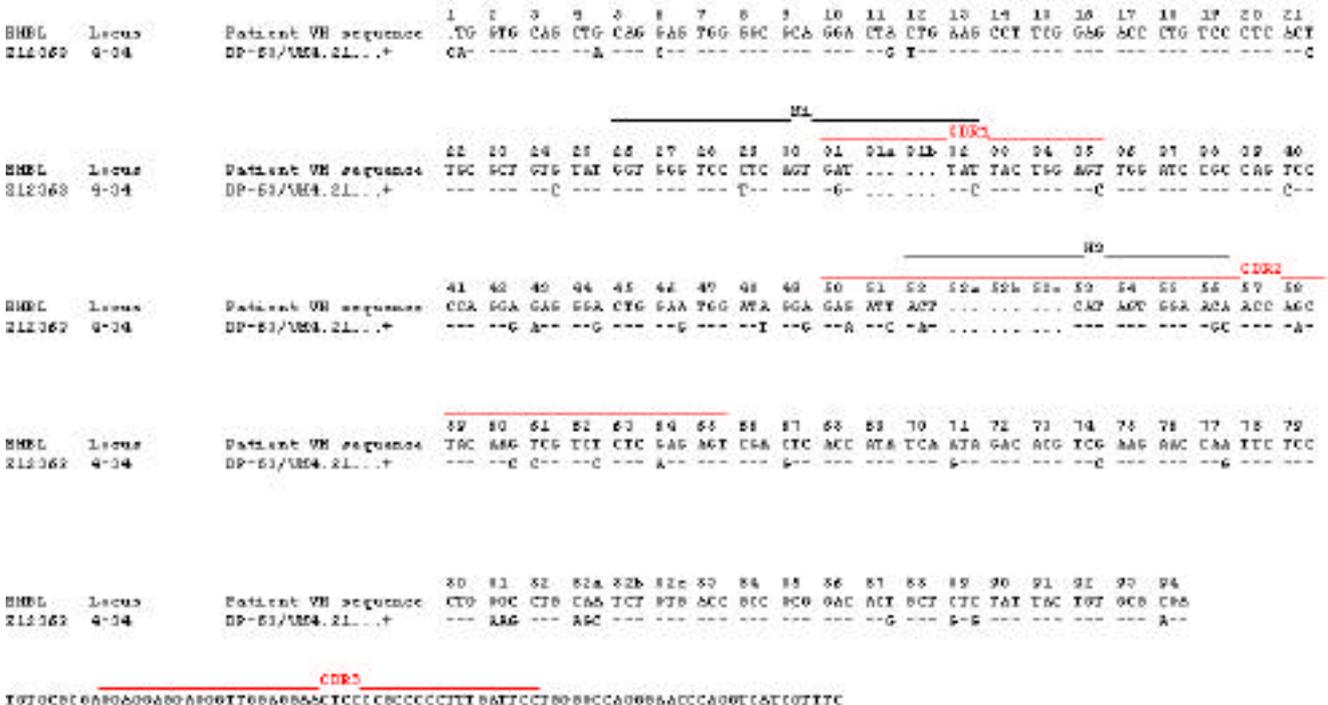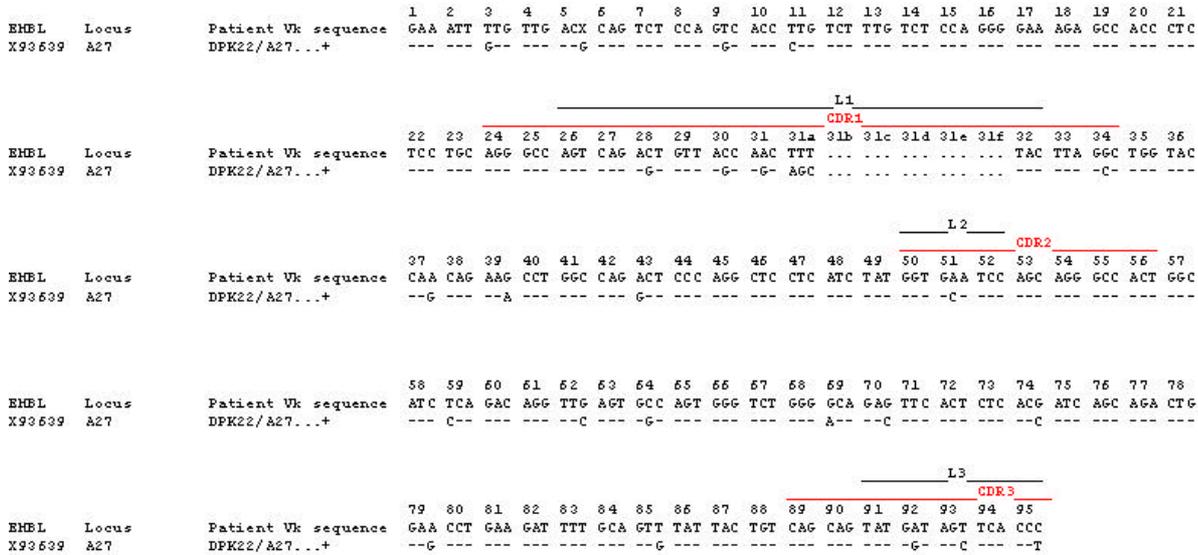
Figure 4. V nucleotide sequence alignment with germline sequence. Representative V sequence of CSF B cells were aligned with the most homologous V germline gene in the database of DNA PLOT. The top line shows the subject's sequence, and the closest germline sequence (DPK22) is shown underneath, where 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 V gene rearrangements and mutations

V					R + S					
				Homology	mutations	R:S	R:S	R:S		
region	CDR3-IgM	Family	Germline	(%)	(total)	(total)	(FR 1+2+3)	(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	
V	QQYDSSPLT	V 3	DPK22	91	20	13:7 (1.86)	6:6 (1)	7:1 (7)		J 1

The IgM VH and V 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 (-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).

References

Galoin S, Daste G, Apoil PA, Chollet F, Roda D, Blancher A, Delsol G, Chittal S, and al Saati T **(1997)** Polymerase chain reaction on cerebrospinal fluid cells in the detection of leptomeningeal involvement by B-cell lymphoma and leukaemia: a novel strategy and its implications. **Br J Haematol** 99, 122-130.

Gleissner B, Siehl J, Korfel A, Reinhardt R, and Thiel E **(2002)** CSF evaluation in primary CNS lymphoma patients by PCR of the CDR III IgH genes. **Neurology** 58, 390-396.

Go JH, Kim DS, Kim TJ, Ko YH, Ra HK, Rhee JC, Kim SW, and Ree HJ **(2003)** Comparative studies of somatic and ongoing mutations in immunoglobulin heavy-chain variable region genes in diffuse large B-cell lymphomas of the stomach and the small intestine. **Arch Pathol Lab Med** 127, 1443-1450.

Hsu FJ, and Levy R **(1995)** Preferential use of the VH4 Ig gene family by diffuse large-cell lymphoma. **Blood** 86, 3072-3082.

Huang N, Kawano MM, Harada H, Harada Y, Sakai A, Kuramoto A, and Niwa O **(1993)** Heterogeneous expression of a novel MPC-1 antigen on myeloma cells: possible involvement of MPC-1 antigen in the adhesion of mature myeloma cells to bone marrow stromal cells. **Blood** 82, 3721-3729.

Hug A, Storch-Hagenlocher B, Haas J, Vogt-Schaden ME, Goldsehmidt H, and Wildemann B **(2004)** Single-cell PCR analysis of the immunoglobulin heavy-chain CDR3 region for the diagnosis of leptomeningeal involvement of B-cell malignancies using standard cerebrospinal fluid cytospins. **J Neurol Sci** 219, 83-88.

Kienle D, Krober A, Katzenberger T, Ott G, Leupolt E, Barth TF, Moller P, Benner A, Habermann A, Muller-Hermelink HK, Bentz M, Lichter P, Dohner H, and Stilgenbauer S **(2003)** VH mutation status and VDJ rearrangement structure in mantle cell lymphoma: correlation with genomic aberrations, clinical characteristics, and outcome. **Blood** 102, 3003-3009.

Longo DL, DeVita, Jaffe ES, Mauch P, and Urba WJ **(1993)** Lymphocytic lymphomas. **Philadelphia, Lippincott.**

Owens GP, Ritchie AM, Burgoon MP, Williamson RA, Corboy JR, and Gilden DH **(2003)** Single-cell repertoire analysis demonstrates that clonal expansion is a prominent feature of the B cell response in multiple sclerosis cerebrospinal fluid. **J Immunol** 171, 2725-2733.

Rhodes CH, Glantz MJ, Glantz L, Lekos A, Sorenson GD, Honsinger C, and Levy NB **(1996)** A comparison of polymerase chain reaction examination of cerebrospinal fluid and conventional cytology in the diagnosis of lymphomatous meningitis. **Cancer** 77, 543-548.

Ritchie AM, Gilden DH, Williamson RA, Burgoon MP, Yu X, Helm K, Corboy JR, and Owens GP **(2004)** Comparative analysis of the CD19+ and CD138+ cell antibody repertoires in the cerebrospinal fluid of patients with multiple sclerosis. **J Immunol** 173, 649-656.

Shimizu S, Tamagawa Y, Kojima H, Mori N, Nagata M, Noguchi M, and Nagasawa T **(2003)** Simultaneous development of lymphoplasmacytic lymphoma and diffuse large B-cell lymphoma--analyses of the clonal relatedness by sequencing CDR3 in immunoglobulin heavy chain genes. **Eur J Haematol** 70, 119-124.

Storch-Hagenlocher B, Haas J, Vogt-Schaden ME, Bentz M, Hoffmann LA, Biessmann A, and Wildemann B **(2000)** Molecular analysis of the CDR3 encoding region of the immunoglobulin heavy chain locus in cerebrospinal fluid cells as a diagnostic tool in lymphomatous meningitis. **Ann Neurol** 47, 211-217.

Szczepek AJ, Seeberger K, Wizniak J, Mant MJ, Belch AR, and Pilarski LM **(1998)** A high frequency of circulating B cells share clonotypic Ig heavy-chain VDJ rearrangements with autologous bone marrow plasma cells in multiple myeloma, as measured by single-cell and in situ reverse transcriptase-polymerase chain reaction. **Blood** 92, 2844-2855.
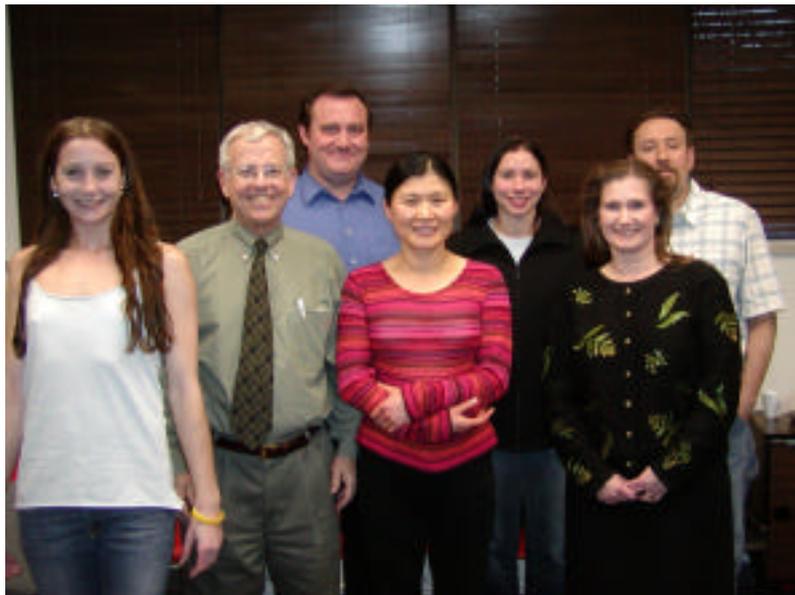
First row from left to right: Kathryne M. Keays, Donald H. Gilden, Xiaoli Yu, Denise M. Damek

Second row from left to right: Mark P. Burgoon; Alanna M. Ritchie, Gregory P. Owens