Primary Central Nervous System Lymphomas Are Derived from Germinal-Center B Cells and Show a Preferential Usage of the V4–34 Gene Segment

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Primary central nervous system lymphomas (PCNSLs) have recently received considerable clinical attention due to their increasing incidence. To clarify the histogenetic origin of these intriguing neoplasms, PCNSLs from 10 HIV-negative patients were analyzed for immunoglobulin (Ig) gene rearrangements. All tumors exhibited clonal IgH gene rearrangements. Of the 10 cases, 5 used the V4–34 gene segment, and all of these lymphomas shared an amino acid exchange from glycine to aspartate due to a mutation in the first codon of the complementarity-determining region 1. No preferential usage of D_H, J_H, V_K, J_K, V_λ, or J_λ gene segments was observed. All potentially functional rearrangements exhibited somatic mutations. The pattern of somatic mutations indicated selection of the tumor cells (or their precursors) for expression of a functional antibody. Mean mutation frequencies of 13.2% and 8.3% were detected for the heavy and light chains, respectively, thereby exceeding other lymphoma entities. Cloning experiments of three tumors showed ongoing mutation in at least one case. These data suggest that PCNSLs are derived from highly mutated germinal-center B cells. The frequent usage of the V4–34 gene and the presence of a shared replacement mutation may indicate that the tumor precursors recognized a shared (super) antigen. (*Am J Pathol* 1999, 155:2077–2086)

the lack of a conventional lymphatic system including lymph nodes and draining lymphatic vessels; and the inability of the CNS to reject allografts, as well as the absence of major histocompatibility antigens on brain cells.^{1,2} Modern neuroimmunology has modified this concept and demonstrated that the immunologically down-regulated phenotype of the brain is partially mediated by an immunosuppressive activity of brain cells^{3,4} and that the CNS is integrated into a complex, tightly regulated neuroimmune network. It is interesting that, although the normal brain is devoid of a significant number of lymphocytes, highly malignant lymphomas may develop in the brain.

Indeed, the incidence of primary CNS lymphomas (PCNSLs) has significantly increased over the last decade.⁵ This entity now accounts for up to 6.6% of all primary malignant intracranial tumors.⁶ An important risk factor for the development of PCNSL is primary or secondary immunodeficiency. HIV-infected patients show a 1000-fold increased risk as compared with HIV-negative persons.^{7–9} However, an increase in the incidence of PCNSL has also been shown in immunocompetent patients, the reason for which is unknown.

Histopathological and immunohistochemical analyses have identified the vast majority of PCNSLs as highly malignant, strongly proliferating B cell non-Hodgkin's lymphomas. They are categorized as diffuse large-cell lymphomas (DLCL) based on the revised European-American lymphoma classification system.¹⁰ It is interesting that PCNSLs differ in their biological behavior from extracerebral DLCL and appear to be associated with significantly reduced survival.⁵ This clinical observation has raised the question whether PCNSLs compose a separate disease entity. In addition, PCNSLs are distinguished from extracerebral lymphomas by the fact that they are strictly confined to the CNS and neither manifest extracerebrally nor metastasize to other organs including the lymphatic system.^{11,12}

The pathogenesis of PCNSL is still poorly defined. Whereas PCNSLs of HIV-positive and -negative patients share many clinical features including their exclusive lo-

The central nervous system (CNS) has traditionally been regarded as an immunologically privileged organ. This view was based on the existence of the blood-brain barrier, which separates the brain from the immune system;

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Table 1. Clinical Data, as Well as Histopathological, Immunohistochemical, and *in Situ* Hybridization Findings

Case number	Age (years)	Sex	Location	EBER [†]	MIB-1 [†]
1	51	f	Temporal	—	+++
2	69	f	Parieto-occipital	±	+++
3	74	f	Occipital	—	+++
4	65	f	Temporal	—	+++
5	65	m	Fourth ventricle*	—	+++
6	73	f	Parietal*	—	++
7	62	f	Frontal	—	+++
8	49	m	Frontal	—	++
9	67	f	Occipital	—	+++
10	61	m	Frontal	—	+++

All tumors were CD20 positive and classified as malignant non-Hodgkin's lymphoma of the diffuse large-cell lymphoma type; only the location of the biopsied lesion is given.

Abbreviations and symbols: f, female; m, male; *, cases with multifocal intracerebral manifestations.

[†]For the analysis of MIB-1 and Epstein-Barr virus (EBER)-positive cells, a grading system was used: —, negative; +, <20% of the tumor cells stained; ++, 20% to 50% of the tumor cells stained; +++, more than 50% of the tumor cells stained; ±, it could not be decided with certainty whether the EBER-positive cells represented tumor cells or were bystander lymphocytes.

calization in the CNS, the underlying pathogenesis may be quite different. The regular association with Epstein-Barr virus (EBV) infection in AIDS-related PCNSL suggests an oncogenic role of EBV-encoded genes.^{11,12} In contrast, there is no evidence for an involvement of EBV in the development of AIDS-unrelated PCNSL.¹¹

To clarify the molecular pathogenesis of PCNSL, the precise characterization of the histogenetic origin and differentiation stage of the tumor cells is a prerequisite. Recently, a series of PCNSLs from both immunocompetent and immunodeficient patients has been shown to express mutations in the 5'-noncoding region of the *bcl-6* gene,¹² a marker for transition of B cells through the germinal center (GC) of secondary lymphatic organs.¹³⁻¹⁵ These data suggested that PCNSL may in part be related to GC B cells. However, the mere presence of a *bcl-6* gene mutation does not distinguish whether the tumor cells correspond to GC or post-GC B cells, ie, memory B cells and plasma cells. To precisely define the cell of origin, a molecular study of immunoglobulin (Ig) gene rearrangements in association with sequence analysis of rearranged V region genes is a suitable tool. Whereas naive IgM⁺ IgD⁺ CD27⁺ B cells express rearranged but unmutated V region genes,¹⁶ somatic mutations are introduced into V region genes during the process of somatic hypermutation,¹⁷ which occurs in the microenvironment of the GC in an antigen- and T cell-dependent reaction.¹⁸⁻²⁰ Thus, GC B cells and their descendants are characterized by somatically mutated V region genes. Cells leaving this anatomical compartment shut down their hypermutation machinery and do not undergo further somatic mutations. Moreover, in somatically mutated V genes, the pattern of somatic mutations can reveal whether the respective B cells were selected for expression of a functional antigen receptor.

Studies on PCNSL have been hampered by the limited amount of tissue available, and, therefore, Ig V region genes have not yet been examined in detail. In extracerebral lymphomas, a preferential rearrangement of *V_H* gene segments was identified for some lymphoid malignancies as compared with normal B cells. For example, an over-representation of the *VH1* family has been reported for chronic lymphocytic leukemia²¹ and of the *VH4*

family for other subtypes of B cell lymphoma,²² and several IgH V gene segments have been associated with autoimmune disorders.²³ A biased usage of gene segments of the *VH4* family has been detected in one study of extracerebral DLCL,²⁴ which, however, was not confirmed in another series of DLCL.²⁵

To precisely characterize the differentiation stage of PCNSL, we investigated a series of 10 PCNSLs from HIV-negative patients by PCR analysis of their V region genes. Our data point to GC B cells as the histogenetic origin of PCNSL.

Materials and Methods

Tumor Samples

This study included PCNSL samples from 10 HIV-negative patients (Table 1). All specimens were from primary tumors, and most were obtained by stereotactic biopsy. Before neurosurgical intervention, all patients were untreated and had received neither corticosteroids nor radiotherapy. Immediately after neurosurgical removal, sections were cut from the tumor, and the tumor samples were stored at -80°C until used for molecular biological analysis. Cryostat sections (10 μm) were cut from the blocks to assure that the frozen tissue contained sufficient numbers of tumor cells.

Histopathology

All tumors were histopathologically classified according to the revised European American lymphoma classification.¹⁰ The diagnoses were based on a combination of routine morphology, which included hematoxylin-eosin and Giemsa stains, and immunohistochemistry. Staining was performed on paraffin sections.

The expression of the B cell-specific antigen CD20 (clone L26, mouse IgG_{2aκ}; Dakopatts, Hamburg, Germany), the leukocyte common antigen CD45RB (clone PD7/26, mouse IgG_{1κ}; Dakopatts), CD45RO (clone UCHL-1, mouse IgG_{2aκ}; Dakopatts), the T cell-specific antigen CD3 (clone A0452, rabbit IgG; Dakopatts), the macro-

phage-specific antigen CD68 (clone KP1, IgG_{1κ}; Dakopatts), and the proliferation-associated nuclear antigen Ki-67 (clone MIB-1, mouse IgG_{1κ}; Dakopatts) were studied by use of an avidin-biotin-peroxidase complex protocol. After incubation with the primary antibody, a biotinylated rabbit anti-mouse or swine anti-rabbit antibody (Dakopatts) was applied to the sections. The sections were then incubated with the avidin-biotin-peroxidase complex (Dakopatts). Peroxidase reaction product was visualized using 3,3'-diaminobenzidine (Sigma Chemical Co., Deisenhofen, Germany) and H₂O₂ as cosubstrate. Sections were lightly counterstained with hemalum. All incubation steps were carried out at room temperature. Control reactions included omission of the primary antibody or application of an irrelevant antibody of the same IgG class instead of the primary antibody.

EBV-Encoded RNA in Situ Hybridization

Small EBV-encoded RNA (EBER) *in situ* hybridization was performed with paraffin-embedded tissue sections as described previously.²⁶ The EBER probes were kindly provided by Dr. G. Niedobitek²⁷ and were digoxigenin labeled using the DIG RNA labeling kit (Roche Diagnostics GmbH, Mannheim, Germany).

DNA Extraction

DNA was extracted from frozen tissue with the Nucleo-Spin Tissue Kit (Clontech Laboratories, Heidelberg, Germany). DNA was dissolved in 40 μl of 10 mmol/L Tris–1 mmol/L ethylenediaminetetraacetic acid buffer (pH 7.6). Of this stock DNA solution, 2 to 5 μl, corresponding to 100 ng of DNA, were used in each amplification reaction.

PCR Analysis

Rearrangements of the Ig heavy-chain locus (*IgH*) were amplified with V_H gene segment family-specific primers as published previously.^{25,28,29} Primers hybridize either to the framework region (FR) I (FRI) or to the leader peptide region of the V_H gene segments. Each of six V_H gene segment families (VH1 to -6) was studied in a separate PCR. As downstream primers, a 3'-JH mix (together with VH-leader peptide region primers)³⁰ or a 5'-JH mix (together with the VH-FRI primers) was used.^{29,31} Taq DNA polymerase (Life Technologies, Eggenstein, Germany) was added before PCR. Amplification was carried out for one cycle at 95°C for 3 minutes, 63°C for 30 seconds, and 72°C for 1 minute, followed by 40 cycles at 95°C for 50 seconds, 63°C for 30 seconds, and 72°C for 1 minute. A final extension step at 72°C for 10 minutes was added.

Rearrangements of the light-chain locus (*Igκ* and *Igλ*, together designated as *IgL*) were amplified with V_κ and V_λ gene segment family-specific primers as published previously.^{25,28,32} The primers hybridize to the FRI of the V_κ or the V_λ gene segments, respectively. As downstream primers, J gene segment-specific primer mixes (5'-Jκ or

~Jλ mix) were used.^{20,32} Each V gene segment family was analyzed in a separate PCR. Cycling conditions for rearrangements of the *Igκ* locus were 95°C for 5 minutes and 80°C for 4 minutes during which Taq DNA polymerase was added, followed by 61°C for 30 seconds and 72°C for 1 minute as the first cycle; and 44 additional cycles at 95°C for 1 minute, 61°C for 30 seconds, and 72°C for 1 minute; plus a final extension step at 72°C for 10 minutes. PCR cycling conditions for rearrangements in the *Igλ* locus were one cycle at 95°C for 3 minutes, 63°C for 30 seconds, and 72°C for 1 minute; followed by 44 cycles at 95°C for 50 seconds, 63°C for 30 seconds, and 72°C for 1 minute; plus a final extension step at 72°C for 10 minutes. Taq DNA polymerase was added before PCR.

DNA extracted from EBV-transformed B cell lines (kindly provided by Dr. J. Irsch, University of Cologne), which are characterized by rearranged V region genes of the *IgH* (VH2–VH5 families), as well as *Igλ* (Vλ1 and Vλ3) or *Igκ* (Vκ1–Vκ4 families) loci, was used as positive control in each PCR. In addition, the *p53* gene was amplified as a cellular control gene (data not shown). As negative control, water instead of tumor DNA was used.

Sequence Analysis of Ig Genes and Cloning of PCR Products

Of each PCR reaction, 10 μl was analyzed on a 2% agarose gel stained with ethidium bromide. PCR products (40 μl) were extracted from a 2% low-melting agarose gel (NuSieve GTG Agarose, Biozym, Hessisch-Oldendorf, Germany) with the QIAEx II Kit (Qiagen, Hilden, Germany) and directly sequenced from both sides with the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer Applied Biosystems, Weiterstadt, Germany) and an automated sequencer (ABI 373 and 377, Perkin Elmer Applied Biosystems).

Sequences were compared with human germline *Ig* gene sequences with the International ImMunoGeneTics³³ and GenBank³⁴ databases and DNAPlot³³ and Blast³⁴ software. Codons were numbered according to Kabat et al.³⁵ D_H gene segments were identified by alignment to germline D_H gene segments and were considered homologous when at least 7 consecutive bp or at least 8 bp interrupted by one point mutation matched the germline sequence.

The ratio of replacement (R) to silent (S) mutations (R/S ratio) within the FRs of V genes was determined. This ratio indicates whether the respective B cell has been selected for expression of a functional antigen receptor within a GC.³⁶ Each single-point mutation in FRI–III of all potentially functional sequences was compared with the respective germline sequence and classified as an R or S mutation. From these data, R/S ratios were calculated.

In addition, PCR products of three PCNSLs (cases 4, 8, and 9) were cloned with the TOPO TA Cloning Kit (Invitrogen, Leek, The Netherlands). From each case, eight clones were sequenced from both sides.

Figure 1. Histopathological characteristics of PCNSL. **a:** Lymphoma cells strongly express the CD20 antigen (CD20 immunostaining, original magnification, $\times 280$). **b:** Characteristic growth pattern of a PCNSL with splitting of the blood vessel walls and a diffuse infiltration of the brain parenchyma. Note the marked edema in the affected brain tissue (H&E staining; original magnification, $\times 220$). **c:** More than 50% of the tumor cells are in a proliferating state as demonstrated by nuclear Ki-67 immunoreactivity (MIB-1 immunostaining; original magnification, $\times 280$). **d:** The tumor cells are embedded in an infiltrate of small, mature T cells, which reside in a perivascular location and are also scattered throughout the brain (CD3 immunostaining; original magnification, $\times 280$).

Results

Neuropathological Analysis

The clinical and histopathologic data of the 10 HIV-negative patients analyzed are summarized in Table 1. At the time of neurosurgical intervention, the patients' mean age was 64 years (range 49–74 years). Eight patients had solitary tumors, whereas two patients presented with multifocal intracerebral lesions. All tumors resided in the supratentorial brain parenchyma. One patient (case 5) had an additional tumor focus in the posterior fossa around the fourth ventricle, and in another patient (case 6), the lymphoma also involved the meninges and extended into the overlying subarachnoid space. None of the patients included in this study had lymphoma manifestation outside the CNS.

Histopathologically (Figure 1), all tumors were classified as CD20⁺ B cell non-Hodgkin's lymphoma of the DLCL type based on the revised European American lymphoma classification.¹⁰ Tumor cells mostly exhibited a centroblast morphology (Figure 1a). The mitotic activity was generally high, and the majority of the tumors contained more than 50% MIB-1-positive proliferating cells (Table 1, Figure 1b). Of 10 PCNSLs, 9 did not harbor EBER; in one tumor (case 2), some small cells appeared

to be EBER positive (Table 1). However, it could not be decided with certainty whether the EBER-positive cells were tumor cells or bystander lymphocytes. In all tumors, the malignant cells were characteristically intermingled with an infiltrate composed predominantly of leukocyte common antigen-positive, CD45R0⁺, CD3⁺ small, mature T lymphocytes, as well as CD68⁺ macrophages (Figure 1c) and some CD20⁺ small, mature, presumably nonmalignant B cells. All lymphomas showed a characteristic angiocentric growth pattern with splitting of the blood vessel walls (Figure 1d). In addition, the tumors diffusely infiltrated the adjacent brain parenchyma, which showed edema and reactive astrocytosis. Areas of necrosis were present in some cases.

IgH and IgL Gene Rearrangements in PCNSL

To characterize the differentiation stage of the tumor cells, 10 PCNSL cases were analyzed for rearrangements of the *IgH*, *IgK*, and *IgL* chain loci by use of six *V_H*, six *V_K*, and eight *V_L* family-specific primers in separate reactions together with the respective *J* gene segment primer mixes. Under these conditions, rearranged *Ig* genes could be identified in all PCNSLs of this series. For the *IgH* gene analysis, one or two dominant PCR products

Table 2. *IgH* and *IgL* Gene Rearrangements in Primary Nervous System Lymphomas

Case number	<i>VH</i>	<i>DH</i> [†]	<i>JH</i>	Mutations [‡] (n)	Mutation frequency [§]	In frame [¶]	<i>VL</i>	<i>JL</i>	Mutations [‡] (n)	Mutation frequency [§]	In frame [¶]
1	V4-34*02	na	J3*02	33	11.2	+	—	—			
2	V3-23*01	D2-2*02	J4*02	11	3.7	+	Vκ1-8*01	Jκ5*01	5	2.5	+
3	V3-23*01	na	J4*02	45	22.2	+	Vκ3-15*01	Jκ4*01	35	13.7	+
4	V3-7*02	na	J4*01	47	16.0	+	Vκ1-8*01	Jκ3*01	55	21.7	—
5	V4-34*02	D3-10*01	J4*02	34	11.8	+	Vκ1-37*01	Jκ4*01	0	0	—
							Vκ2-40*01	Jκ5*01	4	1.5	+
6	V3-30*01	D3-10*01	J3*01	43	14.6	+	Vκ3-11*01	Jκ1*01	44	17.3	+
7	V4-34*02	D3-3*01	J3*01	44	15.6	+	—	—			
8	V4-34*02	na	J5*02	42	14.4	+	—	—			
9	V3-7*01	D3-10*01	J4*02	26	8.9	+	Vλ2-8*01	Jλ3*01	13	4.9	+
10	V4-34*02	na	J6*03	47	16.6	+	Vκ1-5*03	Jκ1*01	24	9.1	+

[†]na, the sequence between the *V_H* and the *J_H* gene segment could not be aligned to any known *D_H* gene segment.

[‡]Values designate the number of point mutations in the *V* gene segment as compared with the most homologous germline sequence.

[§]Ratio between the number of point mutations and the base pair length of the respective *V* gene segment.

[¶]+, in-frame rearrangements; —, out-of-frame rearrangements. All in-frame rearrangements did not contain a stop codon. Accession numbers of the germline gene segments are as follows: *IgH*: V3-7*01, M99649; V3-7*02, X92288; V3-23*01, M99660; V3-30*01, M83134; V4-34*02, M99684; D2-2*02, X97051; D3-3*01, X13972; D3-10*01, X13972; J3*01, J00256; J4*01, J00256; J4*02, X86355; J5*02, X86355; J6*03, X86356. *Igκ*: V1-5*03, X72813; V1-8*01, Z00014; V1-37*01, X59316; V2-40*01, X59316; V3-11*01, X01668; V3-15*01, M23090; J1*01, J00242; J3*01, J00242; J4*01, J00242. *Igλ*: V2-8*01, X97462; J3*01, X97462. Sequences have been submitted to the GenBank data library under accession numbers AF168797–AF168823.

were detected in all tumors (Figure 2). Occasionally, some additional, diffuse bands for other *VH* gene families were disclosed in the agarose gels. These faint PCR products were most likely derived from nonmalignant, polyclonal B cells, which are characteristically part of the accompanying infiltrate.

In each lymphoma, a monoclonally rearranged *IgH* gene could be detected. Among 13 dominant PCR products chosen for sequence analysis, 10 turned out to be clonal *Ig* gene rearrangements, as indicated by clearly readable sequences. Corresponding *V_H* and *J_H* gene segments were identified in all cases. *D_H* gene segments could be identified in five of the tumors. For the light-chain locus, rearranged *V* genes were observed in seven PCNSLs. Three tumors lacked dominant amplicates, and in these lymphomas, neither monoclonal *Igκ* nor *Igλ* gene rearrangements could be detected. This is most probably because highly mutated rearranged gene segments (as they are present in PCNSL; see below) may sometimes not be recognized by the primers.

Sequence Analysis of *V* Region Genes

The successful identification of a clonal cell population allowed us to further examine rearranged *Ig* genes. Results of the sequence analyses are summarized in Table 2. It is interesting that examination of the *VH* gene repertoire revealed a strong bias toward a restricted usage of *VH* gene families. In this series of PCNSLs, only *V_H* gene segments of the *VH3* and *VH4* families were found, each family accounting for 5 of the 10 cases. More important, all monoclonal B cell populations from PCNSLs, which used a gene of the *VH4* family, had rearranged the *V4–34* gene segment (Table 2). Sequence analysis of the 10 cases demonstrated that all of these rearranged *VH* genes were in-frame and did not harbor stop codons, and, thus, they were considered as potentially functional. Remarkably, all PCNSLs using the *V4–34* gene segment shared a common mutation in the first codon of complementarity-determining region 1 (Figure 3). Translation of

the nucleic acid sequence into the amino acid sequence disclosed that this mutation resulted in an exchange from glycine to aspartate.

In addition, five PCNSLs used gene segments of the *VH3* family. Among this subgroup of tumors, two lymphomas had rearranged the *V3–23* gene segment. Another two tumors shared the same *V_H3* germline gene segment, but different alleles (*V3–7*01* and *V3–7*02*). Although both alleles differed at codon 48 (*V3–7*01*, AAG; *V3–7*02*, AAA), they both encoded the amino acid lysine; thus, there was no difference between these two alleles at the amino acid level. The *V3–30* gene segment was used in one case.

In 5 of 10 cases, a *D_H* gene segment could be identified. This was not possible for the other five tumors, because the homology of the sequences to published

Figure 2. PCR analysis of rearranged *IgH* genes. The PCR product of 550 bp indicates the presence of a monoclonal B cell population that has rearranged a gene of the *VH4* family. PCR analysis of *VH* families 3–5 discloses additional faint bands indicating admixed polyclonal, presumably nonmalignant B cells.

Figure 3. Biased usage of the V4-34 gene segment in PCNSL. **a:** The V_H sequences (codons 1-94) of cases 1, 5, 7, 8, and 10 were aligned to the most homologous germline V_H gene segment (V4-34*02). **Dashes** indicate sequence identity. Point mutations are shown either as small letters for silent mutations or as capital letters for replacement mutations. Mutations in codons 93 to 94 of cases 1, 5, and 10 were not taken into account for calculation of the R/S values or the mutation frequency. The **arrow** points to an insertion of 3 bp between codons 27 and 28 in case 1. **b:** Translation of the V_H sequences from **a** and alignment of the amino acid sequences. **Dashes** indicate sequence identity and a **point** indicates a gap of one amino acid for better alignment. **c:** Complementarity-determining region 3 and beginning of FRIV of the IgH sequences (codons 95-103) of five PCNSLs, using the V4-34 gene segment. A sequence comparison demonstrates unique sequences for each case.

germline sequences was too low (less than 7 bp). Three tumors (cases 5, 6, and 9) used the D3-10 gene segment; however, sequence analysis revealed that they had rearranged different parts of the gene segment. The remaining two cases (cases 2 and 7) revealed the D2-2 and D3-3 gene segments, respectively. There was no association with a particular V_H gene segment. J_H gene segments 3 to 6 were found to be rearranged in one (J_H5, 6), two (J_H3), or five cases (J_H4). Again, individual V_H gene segments were not preferentially associated with particular J_H gene segments.

Mutation frequencies of the rearranged IgH genes were calculated. The mean mutation frequency was 13.2% with a range of 3.7 to 22.2%. This frequency significantly exceeds the average mutation frequency of normal, nonmalignant memory B cells (5-6%).¹⁶

Rearranged V_κ genes were amplified from six of seven PCNSLs, which showed a clonal rearrangement of light-chain genes. Two PCNSLs harbored the same V_κ gene segment, V1-8, whereas the other tumors differed in their V_κ gene segments. Thus, in contrast to the V_H gene segments, there was no evidence for a preferential usage of V_κ gene segments in PCNSL. In cases 2, 3, 6 and 10, rearranged Igκ genes were in-frame and did not harbor a stop codon and, therefore, were considered potentially

functional. In one tumor (case 4), a rearranged Igκ gene was detected that was out-of-frame. It contained three stop codons in amino acid positions 16, 37, and 97. It is interesting that tumor 5 harbored two rearranged Igκ genes, one of which was in-frame and one of which was out-of-frame. The out-of-frame Igκ gene was unmutated, and the in-frame Igκ gene showed a low mutation frequency of 1.5%. The absence of somatic mutations in the out-of-frame V_κ gene might be due to an inactivation of the nonfunctional V_κ gene by rearrangement of a κ-deleting element, which often abolishes somatic mutations in remaining V_κJ_κ joints.³⁶ All potentially functional Igκ genes were somatically mutated. The analysis of the J_κ gene segments showed an unbiased usage of the Jκ1, -3, -4, and -5 genes. A clonal, somatically mutated Igλ gene was amplified from one tumor (case 9). This rearrangement was assembled by the gene segments V2-8 and J3, and the rearrangement was potentially functional. The mutation frequency of all potentially functional IgL rearrangements ranged from 1.5% to 17.3% with a mean of 8.3%.

Taken together, sequence analysis of Ig genes of PCNSL demonstrated a biased usage of particular V_H, but not D_H, J_H, V_κ, J_κ, V_λ, and J_λ gene segments. The tumor cells carried a high load of somatic mutations,

a							
codon	24	53	54	57	66	69	92
g1	GCC	GAT	GGA	AAA	CGA	ATC	TGT
3x	---	-T-	-A-	C--	---	---	---
1x	---	-T-	-A-	C--	--g	---	---
1x	---	-T-	-A-	C--	---	---	-A-
1x	---	AT-	-A-	C--	---	---	---
1x	---	-T-	AA-	G--	---	--G	---
1x	-T-	-T-	AA-	G--	---	--G	---
b							
codon	2	17	60				
g1	GTG	ACC	AAC	codon	14	96	
6x	--a	---	---	g1	CCT	CGG	
1x	C-a	---	-T-	6x	---	---	
1x	C-a	--t	-T-	1x	--g	---	
				1x	---	G--	
c							

Figure 4. Intracolon diversity in a subset of PCNSL. PCR products of rearranged *IgH* genes were cloned in cases 4 (**a**), 8 (**b**), and 9 (**c**). Eight separate clones were analyzed in each case. Only those codons are shown in which sequence heterogeneity was observed. **Dashes** indicate sequence identity. Point mutations are labeled either as small letters for silent mutations or as capital letters for replacement mutations. g1, germline sequence.

which indicates that the tumor cells had reached the differentiation stage of GC B cells.

Analysis of the Mutation Pattern

The R/S ratio in FRI-III was calculated for all in-frame *IgH* and *IgL* genes. This parameter indicates whether the corresponding B lymphocyte was selected for expression of a functional antibody.³⁶ The R/S ratios for the FRs were determined as 1.3 (range 0.3–2.2) and 1.3 (range 0–2.5) for the *IgH* and the *IgL* genes, respectively. The R/S ratios clearly indicate that the tumor cells (or their precursors) were selected for antibody expression because selected B cells usually show R/S values below 1.5 for the FRs.³⁶

Intracolon Diversity in PCNSL

The *IgH* gene sequences from one tumor (case 4), which were clearly readable, exhibited a few “double peaks.” To investigate whether this observation might be indicative of intracolon variation, this tumor and two additional tumors (cases 8 and 9) were further analyzed in cloning experiments of the *IgH* PCR products. Eight clones per tumor were sequenced (Figure 4).

In case 4, all clones had 47 identical point mutations, as compared with their respective germline gene segment (V3–7*02). Among these 8 clones (Figure 4a), three sequences did not contain further mutations. The other five clones had introduced additional point mutations into their sequence. Among these, two clones had acquired three identical point mutations, and one of these contained one further sequence change. The remaining three clones harbored one individual mutation each. Surprisingly, the locations of “double peaks” in the sequences derived from direct sequencing were identical in the eight cloned sequences.

In case 8, all clones shared 42 point mutations as compared with the respective germline gene segment (V4–34*02). Six clones had identical sequences, two clones exhibited two additional, identical point mutations, and one of them harbored a third unique point mutation (Figure 4b).

All clones derived from tumor 9 had 26 point mutations in common as compared with the respective germline gene segment (V3–7*01). Six clones had an identical sequence, and the other two clones differed from the main clone by a further introduction of another point mutation, which, however, was different in the two clones (Figure 4c).

To confirm whether these sequence differences between the cloned PCR products were indeed indicative of intracolon diversity or simply reflected *Taq* DNA polymerase errors, we calculated the expected errors for our PCR conditions. Based on a *Taq* DNA polymerase error rate of 10^{-5} /bp and cycle,^{37,38} we expect 1 error/2439 bp. Taking into account the total base pairs obtained from the various sequences (case 4, 2568 bp; case 8, 2640 bp; case 9, 2592 bp), approximately one mutation would be expected to result from *Taq* DNA polymerase error in each of the cases. Because the number of different mutations in case 4 (seven mutations) is well above this threshold, we cannot exclude with certainty that the three mutations (one unique mutation) in case 8 and the two unique mutations in case 9 may be due to a *Taq* DNA polymerase error.

Discussion

The aim of the present study was to define the histogenesis and the molecular phenotype of PCNSL, which develop in an environment that normally contains very low numbers of lymphocytes and actively suppresses the proliferation of lymphocytes.⁴ To address these issues, we sequenced Ig V region genes of the tumor cells from 10 patients. All PCNSLs of our series carried monoclonally rearranged V region genes and, moreover, had introduced somatic mutations into the rearranged Ig genes. These data indicate that PCNSLs are derived from mature B cells, which have experienced an antigen and have undergone T cell-dependent affinity maturation in the microenvironment of the GC of secondary lymphatic organs. Additional cloning experiments in three tumors demonstrated ongoing mutation in at least one case. This also points to GC B cells and not to memory B cells or plasma cells as the cells of origin in at least a fraction of PCNSLs.

This study extends previous data by Larocca et al,¹² who suggested that PCNSL frequently may be related to GC B cells. Their hypothesis was based on the observation of mutations in the 5′-noncoding region of the *bcl-6* gene, which are specifically acquired by B cells at the time of transition through the GC.^{14,15} However, *bcl-6* gene mutations are present in both GC and post-GC B cells, and, therefore, the analysis of Larocca et al¹² did not distinguish between GC and post-GC B cells. Moreover, in the latter study, mutations in the *bcl-6* gene could

be identified in only approximately 50% of PCNSLs, which raised the question whether the origin of PCNSL is heterogenous and includes pre-GC B cells. Because all tumors of our series harbored mutated V region genes, we have no evidence for a subset of PCNSL that have not reached the differentiation stage of GC B cells.

Sequence analysis of the rearranged V genes yielded further insights into the characteristics of the tumor cells. Somatic mutations were observed in all potentially functional *IgH* and *IgL* genes (Table 2). It is interesting that the average mutation frequency was rather high, 13.2% and 8.3% for *IgH* and *IgL* genes, respectively. Previously, a similar mutation frequency was reported for V region genes of extracerebral DLCL.^{25,36} For *IgH*, PCNSL exceeded the mutation frequency calculated for other DLCL outside the CNS. The high frequency of somatic mutations in the V genes of PCNSL can be viewed as a further argument that the tumor cells are indeed derived from GC B cells. This high mutation frequency may be acquired during a prolonged interaction of the tumor cell (or its precursor) in the microenvironment of the GC. A transforming event could have occurred before or during proliferation of the tumor cell in the GC. On one hand, affected clones may have become independent of the microenvironment of the GC initiated by the hypermutation machinery itself via translocation of *Ig* genes²⁹ or by introducing mutations in non-*Ig* genes, eg, *bcl-6*.^{14,15} On the other hand, if a memory B cell, which has already inactivated the hypermutation machinery, served as precursor for PCNSL, one would expect to find mutation frequencies typical for memory B cells (5–6%).¹⁶ Thus, the unusually high mutation frequency of *Ig* genes indicates that the tumor clone is derived from a GC B cell.

To address the issue of ongoing mutation in PCNSL, three PCNSLs (cases 4, 8, and 9) were analyzed in cloning experiments with PCR products of rearranged *V_H* genes. These studies demonstrated intraclonal diversity in at least one case and provide evidence for intracerebrally ongoing mutation of PCNSL. Because ongoing mutation normally requires the presence of both antigen and T cells, it is tempting to speculate that an intracerebral antigen in concert with T cells stimulates further expansion of the tumor clone. The observation of ongoing mutation in the CNS, an extranodal organ devoid of conventional lymphatic structures including GC, was surprising. Although we cannot exclude with certainty that a transforming event took place in the GC of secondary lymphatic organs while the B cell clone still introduced somatic mutations and continued to acquire somatic mutations before leaving the GC, it is unlikely that several different tumor clones leave the GC of lymphatic organs simultaneously and selectively home to the CNS. On the other hand, it is also possible that the transforming event rendered the tumor cells unable to inactivate their hypermutation machinery.

In this study, exclusively genes of the *VH3* and *VH4* families were rearranged. Usage of different *V_H3* genes by five of the lymphomas reflects a normal *V_H* gene usage pattern, because approximately 50% of normal human B cells use genes of the *VH3* family.³⁹ However, the five remaining lymphomas showed a rearranged

V4–34 gene segment, which is used by 4% to 7% of normal B cells in humans.³⁹ This finding lends further support to the hypothesis that intracerebral stimuli direct the *Ig* repertoire and have an important influence on the development and biology of PCNSL. Our data, which were obtained from HIV-unrelated PCNSLs with the morphological characteristics predominantly of centroblasts, are at variance with a recent study of *V_H* genes in the PCNSLs of AIDS patients.⁴⁰ In HIV-related PCNSL of the immunoblastic subtype, evidence for biased usage of particular *VH* families or for intraclonal diversity has not been reported. This difference supports the concept that AIDS-unrelated and AIDS-related PCNSLs represent separate disease entities with distinct pathogenesis.

Surprisingly, all tumors with a rearranged *V4–34* gene segment shared a common mutation in the first codon of complementarity-determining region 1 (Figure 3), which leads to an amino acid exchange from glycine to aspartate. The constant detection of this exchange in all tumors using the *V4–34* gene segment markedly exceeded the expected probability, even considering that the first 4 bp of complementarity-determining region 1 correspond to a mutational hotspot motif.⁴¹ This mutation has also been observed in B cells obtained from 2 of 19 patients with cold agglutinin disease^{23,42} and in 1 of 8 mucosa-associated lymphoid tissue lymphomas.⁴³ However, it appears unlikely that these 4 bp indeed constitute a strong mutational hotspot in *V4–34*, because mutations at this position have been reported in only 10% of somatically mutated *V4–34* gene segments as observed by analysis of cDNA libraries.^{44,45} In our study, this shared mutation suggests antigen-selected maturation of the tumor cells. This notion is further supported by the low R/S ratio, which reflects counterselection of R mutations in the FRs and indicates that the tumor cells (or their precursors) have indeed—at least temporarily—been antigen-selected for expression of a functional antibody. In this regard, PCNSLs behave similarly to extracerebral DLCL.²⁵

Whereas PCNSLs appear to preferentially use the *V4–34* gene, it is controversial whether this holds true for extracerebral DLCL. This gene segment was also rearranged in lymphomas of other entities, including high-grade mucosa-associated lymphoid tissue lymphoma, mantle cell lymphoma, and Burkitt's lymphoma.^{22,43,46} A biased usage of *VH4* genes has been reported in one analysis of extracerebral DLCL,²⁴ but could not be confirmed in another study.²⁵ The apparent discrepancy between the various studies may be explained by the limited number of tumors included and differences in anatomical sites from which the samples were derived. Whether the microenvironment of the CNS favors the observed expansion of lymphomas bearing particular *Ig* genes remains to be elucidated. The high frequency of usage of the *V4–34* gene strongly suggests a functional role of the *Ig* encoded by this gene in the development of PCNSL. Either of two mechanisms may underlie its biased usage, an antigen-driven or a superantigen-fostered expansion of *V4–34*-encoded *Ig*, which may also have autoreactive properties.^{47–50} For normal, nonmalignant B lymphocytes, bacterial and viral proteins have

been described to function as superantigens, eg, staphylococcal proteins^{51,52} and the gp120 protein of the human immunodeficiency virus.^{53,54} Interestingly, a survival-fostering effect has been reported for *VH3* and *VH4*—including *V4–34*—expressing human B cells in response to staphylococcal enterotoxins A and D, which rescued these cells from apoptosis in *in vitro* experiments.^{55,56} In our series, the restricted pattern of a small subset of *V_H* genes, together with a random usage of *D_H* and *J_H* gene segments, would be compatible with a superantigen effect. However, candidates for superantigens involved in the pathogenesis of PCNSL have not yet been identified.

On the other hand, antigens may preferentially stimulate the expansion and intracerebral persistence of B cells, which produce antibodies encoded by the respective *V4–34* gene segment. In this regard, a wide variety of viruses are attractive candidates, including polyomaviruses and herpes viruses, which are known to persist in the brain. It is noteworthy that elevated levels of Ig recognized by the 9G4 antibody, which specifically binds to antibodies encoded by the *V4–34* gene segment,^{23,57} have been reported in association with EBV infections.⁵⁰ However, 9 of 10 of our tumors were EBV negative, which is in line with other studies that have found that PCNSLs in HIV-negative patients do not harbor EBV.^{11,12} Thus, our data do not provide evidence that an EBV infection is pathogenetically involved in the biased usage of the *V4–34* gene segment in these PCNSL.

The identification of GC B cells as the origin of PCNSL in non-AIDS patients and the detailed characterization of their molecular phenotype provide a basis for further detailed studies on the pathogenesis of PCNSL.

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