AIDS Primary Central Nervous System Lymphoma: Molecular Analysis of the Expressed VH Genes and Possible Implications for Lymphomagenesis

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AIDS Primary Central Nervous System Lymphoma: Molecular Analysis of the Expressed V<sub>H</sub> Genes and Possible Implications for Lymphomagenesis

Sylvie Julien,* Mirjana Radosavljevic,* Nathalie Labouret,* Sophie Camilleri-Broet,† Frederic Davi,‡ Martine Raphael,‡ Thierry Martin,* and Jean-Louis Pasquali**

AIDS-associated primary central nervous system lymphomas are late events that have an extremely poor prognosis. Despite different hypotheses, the brain localization of these B cell lymphomas remains an enigma. To better define the cell origin of the lymphomas and the possible role of the B cell receptor (BCR) in the brain localization and/or in the oncogenic transformation, we analyzed the V region genes of the Ig heavy chain expressed by lymphoma cells in five randomly selected patients. After amplifying the rearranged V<sub>H</sub>DJ<sub>H</sub> DNA by PCR, cloning, and sequencing of the amplified products, we observed that: 1) of the five lymphomas analyzed, four were clearly monoclonal; 2) there was no preferential use of one peculiar V<sub>H</sub> family or one peculiar segment of gene; 3) the mutation analysis showed that an Ag-driven process occurred in at least two cases, probably before the oncogenic event; and 4) there was no intraclonal variability, suggesting that the hypermutation mechanism is no longer efficient in these lymphoma B cells. Taken together, our results suggest that distinct Ags could be recognized by the BCR of the lymphoma cells in different patients and that, if the Ags are responsible for the brain localization of these B cells bearing mutated BCR, other factors must be involved in B cell transformations in primary central nervous system lymphoma. The Journal of Immunology, 1999, 162: 1551–1558.

In the past, primary central nervous system (CNS) lymphoma (PCNSL) was considered to be a rare disease; however, its incidence increased dramatically with the outbreak of the AIDS epidemic. It was recently suggested that, if the incidence of PCNSL continued to increase, it could become the most common primary malignant neoplasm of the CNS by the year 2000 (1). The overall survival of patients with AIDS-associated PCNSL remains extremely poor (1). Among AIDS-associated lymphomas that are histologically fairly heterogeneous, AIDS-associated PCNSLs have the peculiarity of a homogeneous histology, usually presenting as large cell immunoblastic plasmocytoid lymphomas of the B lymphocyte lineage (2, 3).

The origins of HIV-associated lymphomas have not been clearly elucidated; however, different factors may contribute to the occurrence of these B cell neoplasias (reviewed in Ref. 4), including EBV, immunodeficiency, chronic antigenic stimulation leading to B cell hyperactivity, and genetic lesions. Focusing on PCNSL, the causative factor EBV is consistently found during PCNSL within the lymphoma cells (5), which is not the case during the heterogeneous systemic AIDS-associated lymphomas. Furthermore, AIDS-associated PCNSLs express Epstein-Barr nuclear Ag-2 and latent membrane protein-1, which are two EBV-transforming proteins that are presumed to be of importance in EBV-induced lymphomagenesis (6–8). The responsibility of the immunodeficiency is clearly evidenced by the known epidemiological association between very low CD4+ T lymphocyte counts and the high risk of development of PCNSL (9); the propensity of these lymphomas to grow in the brain could also be related to an even more depressed immune surveillance in this site. Thus, both EBV and profound immunodeficiency could act in the development of PCNSL, as is the case in lymphomas following drug-induced immunosuppression in transplant patients (10, 11). The role of chronic antigenic stimulation as a possible step in lymphomagenesis (12) with regard to HIV-associated lymphoma is suggested by different indirect arguments: 1) HIV-infected patients have B cell chronic activation that could predispose them to B cell malignancy; 2) such patients often have paraproteinemias that can display anti-HIV activity (13); 3) the anti-HIV and anti-IgG specificities were demonstrated in two IgMs produced by AIDS-associated lymphoma cell lines (14); and 4) it was recently shown that systemic AIDS-associated B cell lymphomas preferentially use IgV<sub>H</sub>4 genes (15). These different arguments are relevant in the case of systemic forms of AIDS-associated lymphomas. In trying to understand the main forces driving the lymphoma process during PCNSL, we must take into consideration the molecular analysis of the V region genes of lymphomatous Ig, which could indicate one of these hypotheses indirectly; if a peculiar Ag is responsible for chronic B cell stimulation and the subsequent transformation, then the V<sub>H</sub> repertoire could be biased, like in AIDS-associated systemic lymphomas, but if EBV as well as profound local immunosuppression is primarily responsible, then the V<sub>H</sub> repertoire should be more diverse. This study was performed to directly address these questions in five random cases of AIDS-associated PCNSL.
Table I. Patient characteristics

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>CD4 Count/mm$^3$</th>
<th>Systemic Opportunistic Infection or Secondary Cancer</th>
<th>CNS Infection</th>
<th>Histology</th>
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<td>BOU</td>
<td>34</td>
<td>&lt;10</td>
<td>Kaposi’s sarcoma</td>
<td>Toxoplasma</td>
<td>IBP$^a$</td>
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<tr>
<td>CEL</td>
<td>34</td>
<td>&lt;10</td>
<td>Pneumocystis</td>
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<td></td>
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<td>Cryptosporidia</td>
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<td>COL</td>
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<td>ND</td>
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<td>CMV pneumonia</td>
<td>IBP</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CMV colitis</td>
<td>HIV Encephalitis</td>
<td>IBP</td>
</tr>
<tr>
<td>CORT</td>
<td>32</td>
<td>&lt;10</td>
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<td>IBP</td>
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<tr>
<td>NIC</td>
<td>50</td>
<td>ND</td>
<td>Atypical mycobacteria</td>
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<td>IBP</td>
</tr>
</tbody>
</table>

$^a$ IBP, immunoblastic plasmacytoma.

Materials and Methods

Patients

Characteristics of the patients are described in Table I. All of these lymphomas were defined as immunoblastic lymphomas with plasmacytic differentiation. The five cases were obtained from an autopsy cohort studied at Pitie-Salpetriere Hospital (Prof. J. J. Hauw, Neuropathology Laboratory, Paris, France).

Clinical information, including age, sex, CD4 cell count, and HIV-associated disorders, was collected for each patient. The autopsy material was classically processed with paraffin-embedding and hematoxylin-eosin and Giemsa stainings. The five cases were classified as immunoblastic lymphomas with plasmacytic features. For each case, a sample was snap-frozen in liquid nitrogen until it was used for molecular analysis. DNA was prepared as described previously (16) using SDS lysis, proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation.

Polymerase chain reaction

Two sets of nested amplifications of the purified DNA were performed in a GeneAmp PCR system (Perkin-Elmer, Foster City, CA). For the first set of six PCRs, we used an external antisense J$_H$ consensus primer and one of each of the six V$_H$ family-specific leader primers; the V$_H$ 1 primer was used to detect the V$_H$ 7 subfamily (see Table II). Primers were used at a 1-$\mu$m concentration. A total of 1 U of Taq polymerase (Perkin-Elmer), Taq buffer, and 100 $\mu$m of deoxynucleoside triphosphates were added to a final volume of 100 $\mu$L. After 2 min of denaturation at 94°C, samples were amplified for 30 cycles; each cycle consisted of 1 min at 94°C, 1 min 15 s at an temperature depending upon the primer sequence (optimized during previous experiments; Table II), and 1 min at 72°C followed by elongation for 10 min at 72°C. A total of 5 $\mu$L of each of the first set of PCR reactions was used to perform a second set of PCR reactions by adding an internal J$_H$ consensus primer and one of six internal V$_H$ family-specific leaders. These primers were designed with restriction sites for subsequent cloning. The amplification products were analyzed on ethidium bromide-stained 1% agarose gels for 45 min at 110 V.

Cloning and DNA sequencing

The PCR products were ethanol-precipitated, digested by Sall and Bsp106, and ethanol precipitated again before ligation into pBluescript (Stratagene, La Jolla, CA) using T4 DNA ligase overnight at 4°C. dsDNA sequencing was performed using the Thermo Sequenase cycle-sequencing kit (Amersham, Buckinghamshire, U.K.) according to the manufacturer’s recommendations.

Somatic mutation analysis

For $n$, the number of random mutations, the number of replacement (R) mutations should equal $0.75 \times n$, and the number of expected silent (s) mutations should equal $0.25 \times n$. Without selection, the R and S mutations would occur in the complementarity-determining regions (CDRs) given the number of total mutations. Assuming that Ig function is maintained, the number of R mutations in the frameworks (FRs) was doubled in the formula to obtain a more accurate $p$ value (17).

Table II. List of the primers used for the two sets of PCR amplification

<table>
<thead>
<tr>
<th>Oligonucleotide Primers (V$_H$DJ$_H$ amplification)</th>
<th>Sequences (5’-3’)</th>
<th>Cycles of PCR</th>
<th>Denaturation (1 min)</th>
<th>Annealing (1 min, 15 s)</th>
<th>Extension (1 min)</th>
</tr>
</thead>
<tbody>
<tr>
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<td>V$_H$6</td>
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<td>3’ Antisense: J$_H$ consensus</td>
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<td>Second cycle of PCR: internal primers</td>
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<td>CGAAGCTTGTGCAG CGTTGCAG</td>
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</table>

* Restriction sites are underlined (ATCGAT, CPal; GTCGAC, SaPl).
Results

Analysis of Ig V<sub>H</sub> gene subgroups

PCR-amplified genomic DNA was analyzed in ethidium bromide agarose gels. The results are shown in Fig. 1. In four cases, only one ~520-bp PCR product was generated using oligonucleotides specific for J<sub>H</sub> and the leader sequence of each Ig V<sub>H</sub> family; two lymphoma samples used V<sub>H</sub> 4 genes (COL, CORT), one used a V<sub>H</sub> 5 gene (BOU), and one used a V<sub>H</sub> 1 gene (CEL). Alternatively, PCR with DNA of NIC generated two ~520-bp PCR products (V<sub>H</sub> 1 and V<sub>H</sub> 6). Taken together, the results indicate a heterogeneous use of Ig V<sub>H</sub> genes by lymphomatous cells originating from different patients.

Molecular analysis of the lymphoma-rearranged V<sub>H</sub> region genes

The PCR products of the V regions were cloned into pBluescript, and multiple clones were randomly sequenced. The nucleic acid sequences of each PCR product are given in Fig. 2, A–F, and Fig. 4. CDR3 analysis confirms the monoclonality of the PCNSL cells in four of five patients as well as the oligoclonality in the fifth patient (NIC). All of the sequences obtained represent a functional VDJ rearrangement.

Comparison of the sequences with the most homologous germ-line V<sub>H</sub> genes may give an indication regarding a putative Ag-driven process that occurred before the oncogenic transformation. The homology search was performed with the International Immunogenetics (IMGT) database (18), and the results are presented in Table III. Some of the V<sub>H</sub> are very close to the putative germline (NIC V<sub>H</sub> 1, 99.3% homology with DP15); others differ further from the candidate germline: COL V<sub>H</sub> 4 has 93% homology with an allelic variant of DP63 (VH4-34*03), and CEL V<sub>H</sub> 1 has 93.9% homology with an allelic variant of DP10 (VH1-69*01). Because most human V<sub>H</sub> genes and their allelic variants have now been identified and included in the IMGT database (19), it is highly probable that these differences represent somatic mutations. Because the introns located between the leader and the V<sub>H</sub> regions are not as highly conserved as the coding regions and can be used to identify the germlines (20), we have sequenced the introns of the CEL and COL V<sub>H</sub> rearranged genes to verify this possibility (Fig. 3). The homology search performed with these sequences and two different databases (IMGT and GenBank) confirmed the initial identification of the putative germline: only one (COL) and two (CEL) changes were observed in these noncoding regions, which were also subjected to the mutation machinery. To interpret the significance of these V region mutations, we applied the binomial model-derived probabilities (see Materials and Methods) to show that the distribution of the mutations was not random in the case of patients CEL and COL, clearly suggesting an Ag-driven selection
A. D3 (VH2,5)

| Glu     | Val     | Lys     | Val     | Gln     | Ser     | Gly     | Ala     | Gln     | Val     | Lys     | Pro     | Gln     | Ser     | Leu     | Arg     | Ile     | Ser     | Cys     | Lys     | Ser     | Gly     | Ser     | Gly     | Ser     |
|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
| VH-13   | GIA     | CIG     | CTO     | OTO     | CIG     | GIA     | CSG     | CIG     | GIA     | CIG     | GIA     | CSG     | CIG     | GIA     | CSG     | CIG     | GIA     | CSG     | CIG     | GIA     | CSG     | CIG     | GIA     | CIG     | CSG     | CIG     |
| VH-2    | CIG     | CIG     | GIA     | CSG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     |
| VH-3    | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     |
| VH-4    | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     | CIG     |

B. D4 (VH2,1)

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C. D5 (VH4,4)

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<td>VH-1</td>
<td>DP1</td>
<td>VH-1</td>
</tr>
</tbody>
</table>

FIGURE 2. Ig VH gene nucleotide sequences and intraclonal diversity of the rearranged Ig VH gene cloned from CNS lymphoma. Each sequence is compared with that of the most closely homologous germline Ig VH sequence. Each sequence was designed with the three-letter code name of each patient and assigned a number corresponding with an individual pBluescript clone. CDR3 is represented in Fig. 4 (except for the NIC Ig VH sequences homologous to the VH6 gene) to show that the corresponding B cells were polyclonal. Nucleotide similarities are indicated by periods (.), S mutations are indicated by small letters, and R mutations are indicated by capital letters. The locations of the CDRs are indicated. D gene assignment performed according to Sanz (23).
<table>
<thead>
<tr>
<th>Column 1</th>
<th>Column 2</th>
<th>Column 3</th>
<th>Column 4</th>
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**Figure 2 (continued)**
Table III. Analysis of mutations in PCNSL-expressed V\textsubscript{H} genes\textsuperscript{a}

<table>
<thead>
<tr>
<th>V\textsubscript{H} Gene (% homology)</th>
<th>Location</th>
<th>No. Observed</th>
<th>No. Expected</th>
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<td>FR</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>CDR</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>CORT: DP70 (97.3%)</td>
<td>FR</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>CDR</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>NIC: DP15 (V\textsubscript{H}1) (99.3%)</td>
<td>FR</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>CDR</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

\textsuperscript{a} The germline Ig V\textsubscript{H} genes having the highest homology to lymphoma-isolated sequences are presented using the revised nomenclature for germline Ig V\textsubscript{H} genes. The column marked "% homology" provides the percentage of nucleotide sequence homology between the expressed Ig V\textsubscript{H} gene and its putative germline counterpart. FR indicates the lines providing the analysis of segments FR1, FR2, FR3; CDR indicates the lines providing the analysis of CDR1 and CDR2. Columns marked "R" and "S", indicate the number of deduced R mutations and S mutations in each region, respectively. The columns "No. Observed" and "No. Expected" indicating the observed and expected numbers, respectively, of R and S mutations in the CDRs and FRs. \( p_{\text{CDR}} \) is the probability that an excess of R mutations in the CDRs resulted by chance.

(Table III). This result was confirmed using a modification of the calculation introduced by Chang and Casali (21). For the other number of mutations was too low for us to come to a conclusion.

The patient NIC merits further discussion: two types of V\textsubscript{H}DJ\textsubscript{H} rearrangements were detected (V\textsubscript{H}1 and V\textsubscript{H}6). Because all of the V\textsubscript{H}1 sequences were identical, they most likely correspond with the heavy chain rearrangement expressed by lymphomatous B cells. In contrast, the four V\textsubscript{H}6 analyzed sequences were derived from three different B cell clones in view of CDR3. We could consider that these V\textsubscript{H}6-expressing B cell clones are innocent bystander B lymphocytes present in the biopsy specimen. However, if this hypothesis is correct, it is surprising to find only V\textsubscript{H}6-expressing B cells in contact with the tumor cells, because the V\textsubscript{H}6 gene is unique in humans, rarely used in adult B cells, and rarely mutated (22).

CDR3 is composed of 11–29 amino acid residues (Fig. 4). Assignment of the D gene segment usage was performed using the criteria proposed by Sanz (23). COL and CORT CDR3 probably arose through D-D fusion; the D gene used by CEL could not be identified. Comparison of CDR3 and FR4 (data not shown) with known J\textsubscript{H} segments indicated that BOU and CEL probably use J\textsubscript{H}5, that COL uses J\textsubscript{H}4, and that CORT and NIC V\textsubscript{H}11 use J\textsubscript{H}6.

To evaluate intraclonal variability, we compared randomly selected sequences (shown in Fig. 2, A–F); there was no difference observed between the sequences of the patients CORT and CEL, one nucleotide change in one of the four sequences in patients BOU and COL, and one or two changes in patient NIC V\textsubscript{H}1. The very low frequency of these base changes is compatible with the known error rate of the enzyme Taq polymerase.

Discussion

We report the first molecular analysis of the V\textsubscript{H} genes expressed by AIDS-associated PCNSL. This analysis confirms the monoclonality of the B cell proliferation without ambiguity in four of five patients. The results concerning the fifth patient (NIC) are more difficult to interpret. Two different V\textsubscript{H} families (V\textsubscript{H}1 and V\textsubscript{H}6) were amplified from biopsy material. The sequences originating from the V\textsubscript{H}1 material were identical and are thought to represent the product of the monoclonal B cell lymphoma. On the contrary, the V\textsubscript{H}6 rearrangements were in general distinct from each other and were consequently expressed by different B cells. As stated above, the presence of these cells in contact with the tumor cells is intriguing: 1) If these cells are bystander B cells, other V\textsubscript{H} families that are more frequently expressed in adult B cells should have been detected; 2) if they are not bystander B cells, the possibility that their presence is related to a specific Ag should be considered, which in turn could be assigned to the V\textsubscript{H}6 product (superantigen effect?).

It was recently reported that AIDS-associated systemic B cell lymphomas (10 cases) expressed a restricted set of V\textsubscript{H} genes that primarily belong to the V\textsubscript{H}4 family (15). Our data on AIDS-associated PCNSL give us a more diverse representation of the V\textsubscript{H} family. Without significant bias toward a peculiar V\textsubscript{H} family. However, we were quite surprised to find that we did not detect any V\textsubscript{H}3-containing rearrangement expressed by the lymphoma cells despite the fact that the V\textsubscript{H}3 genes encode most of the Ig expressed by normal adult B cells (21) and that the V\textsubscript{H}3 gene products could have a distinctive ability to bind to HIV glycoprotein 120 (24). It is still unclear whether this binding capacity of the
VH3 gene products is responsible for a selective depletion of VH3-expressing B cells in HIV-infected patients (25–27), but this mechanism could account for the apparently low representation of the VH3 genes during AIDS systemic B cell lymphomas (15) as well as during PCNSL. However, published cases reporting on the occurrence of VH3 expressing AIDS-associated Burkitt’s lymphomas should also be mentioned (28, 29). The mutation analysis of our cases was possible, even though we did not isolate the respective germline genes from these patients. Indeed, it is believed that most human VH genes have now been identified, and that individual polymorphism is generally low (19). In two of the five patients, we found a statistically significant preference for R mutations in the VH CDRs compared with the FRs (binomial model). The accumulation of such mutations in normal B cells generally indicates that the cell of origin migrated through a germinal center, where it was subjected to antigenic contact and where the hypermutation mechanism operated (30, 31). This is also an indirect argument suggesting that the Ag plays a role in the selective process operating in a B cell population. The low degree of mutations in the VH of the remaining three patients prevents any statistical analysis but does not exclude the possibility that the B cell of origin was also driven by antigenic exposure. The analysis of CDR3 provides additional evidence for a possible selection. All CDR3 have nonconservative base differences from the deduced D and JH segments. Moreover, there is a preferential use of long CDR3 (mean = 19 vs 13 for the CDR3 used by unselected normal adult B cells) and of D-D fusions (only 10% of Ig heavy chain CDR3 expressed by normal adult B cells seems to arise by D-D fusion). It is noteworthy that both of these features were repeated by Kipps (15) in AIDS-associated systemic B cell lymphoma. The almost complete absence of ongoing mutations in the lymphoma cells could suggest that PCNSL does not derive directly from germinal center B cells. However, Larocca et al. recently described mutations in the 5' region of the bcl-6 gene in some AIDS-associated PCNSL cells; these mutations are considered to be an argument for either the germinal center or the postgerminal center B cell origin (32). Further work will be necessary to elucidate this point. Thus, the cell of origin more likely matured in the periphery, at least in the patients CEL and COL, and then migrated to the brain. Because these patients do not present any evidence of lymphoma in the periphery, it is reasonable to speculate that the last transforming event(s) took place in the CNS. The responsibility of the depressed immune surveillance in this site is frequently suggested, but the HIV-infected tissue microenvironment could also play a direct role in the initiation of lymphomagenesis.
In the case of AIDS-associated PCNSL, what could be the antigenic contact? The answer to this question is still open to interpretation, because numerous pathogens can be found both in the brain and in the periphery of patients with AIDS including HIV, CMV, EBV, and Toxoplasma gondii. The findings of Ng et al. (14) show that two IgMs produced by AIDS lymphoma cell lines are able to react with HIV glycoprotein 160 or human IgG, suggesting a role for chronic antigenic stimulation during the process of lymphomagenesis. This type of mechanism is best exemplified in mucosa-associated lymphoid tissue gastric lymphoma, in which the growth of the transformed B cells is dependent upon the presence of Helicobacter pylori (33–35). Whether this hypothesis is of importance during AIDS-associated PCNSL is unclear and merits further evaluation: even though the expressed VH genes are diverse, the mutation analysis in two of the five cases and the known importance of both the stochastically derived heavy chain CDR3 (36, 37) and the somatic mutations in determining Ab specificity encourage further studies to address this issue.

References