From brain to testis: immune escape and clonal selection in a B cell lymphoma with selective outgrowth in two immune sanctuariesy

We describe a patient with a primary diffuse large B-cell lymphoma of the central nervous system who developed a localized testicular relapse after 8 years. Both tumours lacked HLA-DR expression, the relapse additionally lost HLA class I expression. Immunoglobulin heavy chain gene rearrangements were identical in both lymphomas with extensive and ongoing somatic hypermutations resulting in extensive idiotype modulation. We hypothesize that these immune sanctuaries initially provided a *safe haven* for the tumour cells. When the environment becomes more permissive for an anti-tumour response, the continuous idiotype modulation and progressive loss of HLA expression on the tumour cells facilitates further immune escape.

Haematologica 2007; 92:(6)e69-e71

Primary central nervous system lymphoma (PCNSL) and lymphoma of the testis are rare forms of diffuse large B cell lymphoma (DLBCL). Both lymphoma types are immune-privileged site-associated DLBCL (IP-DLBCL), are EBV negative (except for immune-compromised patients), and almost exclusively have an activated B cell-like (ABC) phenotype.[1,2] Lymphomas are considered to develop and progress in a multistep manner due to accumulation of genetic aberrations. Where a lymphoma is subject to selective pressure of the immune system, such aberrations may give rise to an *immune escape* phenotype.[3] A common aberration leading to immune escape of PCNSL and testicular DLBCL is loss of HLA expression.[4,5,6] Another common feature of both lymphoma types is a high level of (often ongoing) somatic hypermutation (SHM) in the immunoglobulin heavy chain (IgH) genes.[1,7,8]

Although usually presenting with stage IE disease, patients with PCNSL or testicular DLBCL have a poor prognosis that has only recently improved upon introduction of novel chemotherapy strategies.[9,10] Primary testicular DLBCL frequently relapse in the CNS up to 10 years after initial presentation.[10,11] Relapse of PCNSL is almost always (90-95%) confined to the CNS. One patient has been reported with a relapse of PCNSL in the testis, accompanied by extensive systemic involvement,[12] but no patients have been described with a relapse solely in the testis. We describe a unique patient having a CNS lymphoma with a relapse confined to the testis 8 years after diagnosis. The ongoing modulation of the idiotype, in addition to progressive loss of HLA class II and I proteins, might have provided an efficient tumour escape mechanism in these lymphomas.

Materials and methods

Histology

Formalin-fixed paraffin-embedded material of both CNS and testicular lymphomas was available. Staining with anti-CD20 (L26, DAKO, Glostrup, Denmark) confirmed the B cell origin. Stainings with anti-CD3 (PS1, Monosan, Uden, The Netherlands), anti-CD8 (C8/144B, DAKO) and anti-CD68 (PG-M1, DAKO) were performed to determine the presence of T cells and macrophages in the tumour microenvironment. Stainings with anti-Bcl6 (PG-B6p, DAKO), anti-CD10 (56C6, Novocastra, Newcastle upon Tyne, UK) and anti-MUM1/IRF4 (MUM1p, DAKO) were considered positive if more than 30% of neoplastic cells were stained. Stainings with anti-HLA-A/G, anti-HLA-B/C (HCA2 and HC10; Dr. J. Neefjes, NKI, Amsterdam) and anti-HLA-DR (LN3, Biotest AG, Dreieich, Germany) were considered negative when staining of tumour cells was absent in the presence of a positive internal control. EBER *in situ* hybridization for EBV was performed according to manufacturer's protocol (DAKO).

Immunoglobulin mutation analysis

DNA was isolated from paraffin tissue and IgH multiplex PCR and GeneScan analysis were performed as described.[1] Unlabeled PCR products were cloned and from both tumours 20 colonies (10 for each duplicate PCR) were sequenced. Sequences were analyzed using IMGT/VQUEST13 and aligned using ClustalW14 and were deposited in GenBank (EF205597 to EF205627).

Results

In March 1996 a male patient, age 60, was diagnosed with a stage IE, right temporal PCNSL. Complete remission was achieved with chemotherapy (alternating high doses of MTX/Teniposide and high doses of MTX for 4 weeks, with 4x intermittent intrathecal MTX), followed by radiotherapy (40 Gy in 20 fractions). In June 2004, the patient presented with a tumour of the left testicle and a diagnostic orchidectomy revealed DLBCL. Restaging disclosed stage IE disease. The patient was treated with 4x CHOP with intrathecal MTX and radiotherapy of the left groin area and scrotum (30 Gy in 15 fractions). In August 2005, the patient developed neurological symptoms, most probably due to post-radiation encephalopathy. Until September 2006 no disease activity was found. Both PCNSL and testicular DLBCL were CD20 positive and EBV negative. The PCNSL was heterogeneous for CD10, negative for Bcl6 and positive for MUM1; the testicular DLBCL was negative for CD10 and positive for Bcl6 and MUM1, compatible with an ABC-like immunophenotype.[1] Both localisations showed loss of HLA-DR expression, the testicular DLBCL showed additional loss of HLA class I expression. Very few T cells (including CD8-positive cytotoxic T cells) and macrophages were present in the micro-environment of the CNS lymphoma, while the numbers for both cell types were higher in the micro-environment of the testicular lymphoma. The T cells that were present were mostly CD8 positive.

GeneScan analysis of IgH rearrangements showed identical rearrangements in both localisations, confirming that the testicular DLBCL was a relapse from the PCNSL. Sequence analysis revealed 31 clones derived from the same rearrangement (C1-C18 and T1-T13). CDR3 V-D-J junctional sequences showed highest homology to IGVH3-30*18, DH2-2 and JH5*02. A hypothetical consensus sequence, *shared CNS*, was considered as PCNSL founder clone and carried 52 mutations (23%) compared to the germline V3-30*18 allele, of which 35 were located in a mutation hotspot or a directly adjacent codon (Figure 1). Using the multinomial model15, significant negative selection pressure on the framework regions was observed ($p \leq 0.005$). Considerable intraclonal heterogeneity was found at both lymphoma sites (Supplementary Figure 1).

Discussion

We describe a unique patient with primary DLBCL of one immune sanctuary (CNS) and a very late, isolated relapse to another immune sanctuary (testis). Both localisations were clonally related, and showed a high level of ongoing SHM and progressive loss of HLA expression.

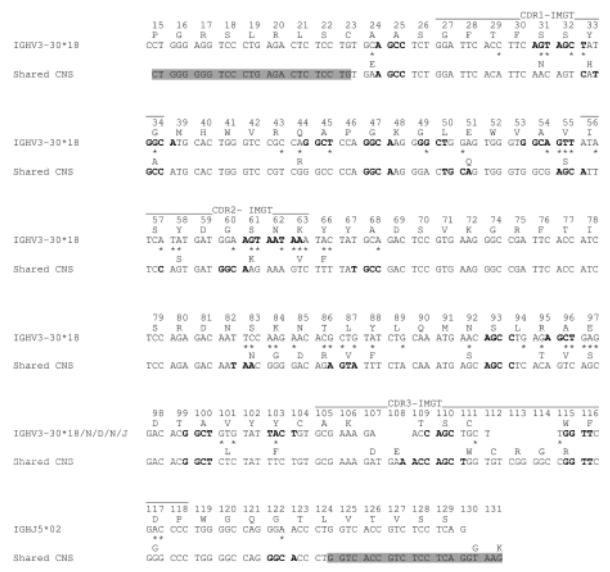


Figure 1. Alignment of hypothetical IgH consensus 'shared CNS' sequence with germline V3-30*18, D2-2 and J5*02 allele sequences. Codon numbering according to IMGT[13]. Mutations in the 'shared CNS' consensus are indicated by asterisks under the germline sequence. Mutation hotspots (RGYW, WRCY and TAA motifs) are indicated in bold. Primer sequences are highlighted in grey. The complete alignment of all lymphoma sequences is available as Supplementary Figure 1.

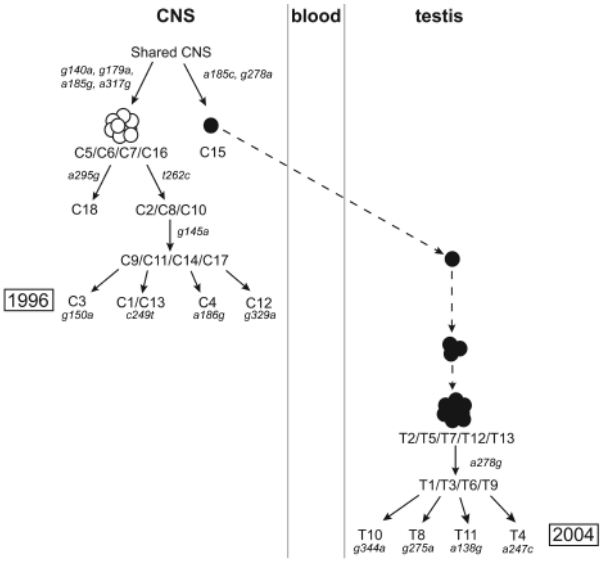
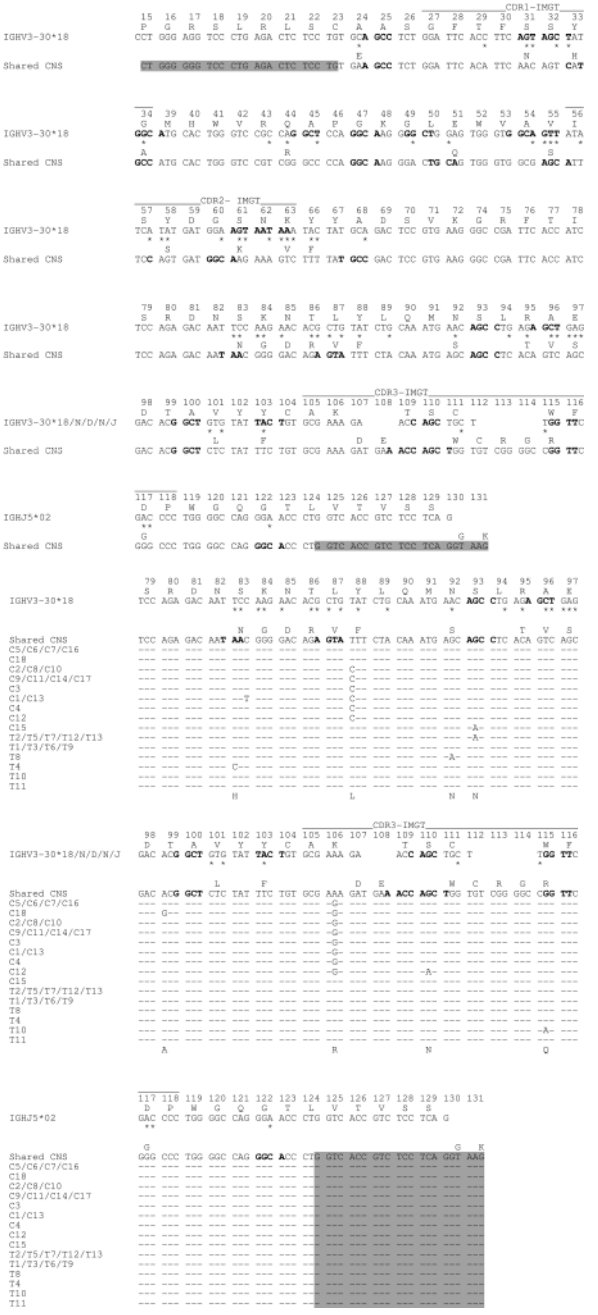


Figure 2. Hypothetical model of the development of the PCNSL and its subsequent relapse in the testis. At a certain point in PCNSL development (in or before 1996), ongoing hypermutation of a 'shared CNS' lymphoma clone resulted in 2 subclones (represented by open and filled cells respectively). While the major subclone (open cells) continued to diversify within the CNS environment, the minor subclone (filled cell) migrated through the bloodstream to the testis. Here it possibly stayed clinically dormant for many years to finally result, after 8 years, in a clinically manifest lymphoma (filled cells), which continued to accumulate mutations. In this figure mutations of individual clones relative to the consensus *shared CNS* sequence are indicated by the mutated nucleotide number.



Supplementary Figure 1. Alignment of lymphoma IgH sequences with germline V3-30*18, D2-2 and J5*02 allele sequences. C: sequences from PCNSL, T:sequences from testicular DLBCL. Codon numbering according to IMGT13. The sequence of the hypothetical consensus 'shared CNS' is included and mutations in this consensus are indicated by asterisks under the germline sequence. Additional mutations in lymphoma sequences are indicated relative to this consensus sequence. If an additional mutation in a lymphoma clone leads to an amino acid replacement compared to the consensus sequence, the new amino acid is indicated below the sequence of T11. Mutation hotspots (RGYW, WRCY and TAA motifs) are indicated in bold. Primer sequences are highlighted in grey.

The observed SHM frequencies are considerably higher than in normal GC and post-GC B-cells[16] and nodal DLBCL.[17] but comparable to the high SHM frequencies that are common in DLBCL of the testis and CNS.[1,7,8]

We used IgH SHM analysis to deduce a model for the development of these lymphomas (Figure 2). Since there was a common tumour subclone between both localiza-

tions (C15 and T2/T5/T7/T12/T13 were identical), we considered a subclone selection model as the most appropriate.[18] According to this model, PCNSL subclone C15 founded the testicular lymphoma. This model fits with the clinical presentation (the testicular lymphoma developed after the CNS lymphoma). None of our sequences contained nonsense or frameshift mutations. Moreover, all sequences had a significantly lower than expected R/S ratio in the framework region indicating maintenance of the overall structure of the functional B cell receptor and the presence of selection pressure. This is reminiscent of the oligoclonal B cells from the CSF of patients with MS with a high load of ongoing SHM and strong preservation of the FR regions,[19] and CSF derived B-cells implicated in the generation of autoantibodies against GM1 gangliosides in neuropathy.[20] A BLAST analysis of the PCNSL CDR3 sequence, as described before for MALT lymphomas,[21] did not reveal any homology to antibodies directed against known autoantigens (*data not shown*). Based on the results from the current study and previous reports we propose a hypothesis for the biological behaviour of immune-privileged site-associated DLBCL. Both PCNSL and testicular DLBCL have IgH open reading frames with an extremely high load of ongoing somatic mutations, many leading to amino acid and idiotype changes.[1,7,8] In immune sanctuaries, a delicate balance exists between a tolerant/inhibitory immune response and an active cytotoxic immune response. Several mechanisms act together to provide an environment in which this balance is skewed towards tolerant or inhibiting responses.[22,23] We hypothesize that the high load of mutations makes the tumour cells highly immunogenic and subject to an anti-idiotype immune response, and that in consequence the tumour cells initially can only survive within an immune sanctuary where this response is absent. When subsequently these lymphomas start to grow, the balance will eventually be disturbed, rendering the environment more permissive for an anti-tumour cytotoxic immune response. This is substantiated by high numbers of infiltrating cytotoxic T cells in PCNSL and testicular DLBCL at the moment of clinical diagnosis.[6] In the case presented here, this infiltrate is more pronounced in the testicular relapse than in the CNS localisation. Under pressure of this immune reaction the ongoing remodelling of the idiotype, in addition to progressive loss of HLA class II and HLA class I expression, might thus provide escape mechanisms for the tumour cells, necessary for their sustained survival and growth at these sites.

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