The antigen receptor in the pathogenesis of B-cell non-Hodgkin's lymphomas
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Primary cutaneous B-cell lymphomas are B-cell non-Hodgkin's lymphomas that arise in the skin. The major subtypes discussed are follicle center cell lymphomas, immunocytomas (marginal zone B-cell lymphomas), and large B-cell lymphomas of the leg. In this study, we analyzed the variable heavy chain (VH) genes of these lymphomas, i.e., follicle center cell lymphomas (diffuse large-cell lymphomas) and 3 immunocytomas. We show that all these lymphomas carry heavily mutated VH genes, with no obvious bias in VH gene usage. The low ratios of replacement versus silent mutations that arise in the skin, with no evidence of extracutaneous disease for a period of at least 6 months after diagnosis.

Organisation for Research and Treatment of Cancer (EORTC) has proposed a new classification based on clinical, histologic, and immunological criteria. The major subtypes are primary cutaneous follicle center cell lymphomas and immunocytomas (marginal zone B-cell lymphomas), both of indolent clinical behavior. Another group of PCBLs, i.e., large B-cell lymphomas of the leg, is considered to be of intermediate malignancy. Two provisionally designated entities are intravascular large B-cell lymphomas and plasmacytomas.

Although the nomenclature of PCBLs suggests an established relationship with nodal B-NHL counterparts and/or with differentiation stages of normal B-cell ontogeny, virtually no data are as yet available on the composition of the B-cell antigen receptors (BCR) of PCBLs. Normal B-cell maturation is characterized by stepwise alterations of the BCR. Naive B cells carry unmutated Ig variable heavy chain and light chain genes that are expressed at the cell surface as IgM and IgD isotypes. In germinal centers of secondary follicles, B cells proliferate and compete to bind antigens that are exposed at the surface of follicular dendritic cells (FDCs). Recognition of antigen elicits signals essential for proliferation and differentiation. During the subsequent cell divisions, somatic mutations are introduced in the variable Ig genes (reviewed by Kock's and Rajewsky's). Because of strict selection processes, the germinal center reaction finally yields B cells with no normal patterns of somatic mutations and augmented affinity for the recognized antigens. These post-germinal center cells, either memory B cells or plasma cells, often express heavy chain isotypes other than μ and δ, which has implications for the effector functions of the secreted Ig.

To obtain information on the maturational state of PCBLs, we analyzed the variable heavy chain (VH) region genes of 7 of these lymphomas. We show that they all carry significantly mutated VH genes with mutation patterns reminiscent of antigen selection processes. This finding indicates that PCBLs are derived from germinal center cells or their descendents. Moreover, evidence was obtained for ongoing somatic hypermutation and isotype switching, features that are shared with extracutaneous B-NHLs of mucosa-associated lymphoid tissue (MALT) and follicular lymphomas.

**MATERIALS AND METHODS**

**Patient material**. Tissue material of 7 PCBLs, i.e., follicle center cell lymphomas, 3 immunocytomas (marginal zone B-cell lymphomas), and 1 polylymphoma, was obtained from the Departments of Dermatology and Pathology of the Free University Hospital and the Department of Pathology of the Academic Medical Center (Amsterdam, The Netherlands). The diagnoses were based on the characteristic clinical and histologic criteria, described previously. The follicle center cell lymphomas showed a predominance of centroblasts and are further referred to as diffuse large B-cell lymphomas. Follow-up data confirmed the favorable prognosis of these lymphomas, with all patients alive and in complete remission 12 to 106 months after diagnosis. The clinical and histological data of the 8 patients are summarized in Table I.

**Immunohistochemistry**. The expression of surface Ig isotype, CD70, and the presence of FDCs was determined immunohistochemically on cryostat sections. Monoclonal antibodies specific for human Ig isotypes and CD21 (DRC-1) were purchased from DAKO (Glostrup, Denmark), except for anti-IgM, which was obtained from Becton Dickinson (Fremboegem-Aalst, Belgium). CLB-CD70-1, specific for CD70, was a kind gift from Dr R.A.W. van Lier (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands). Acetone-fixed tissue sections were preincubated with 10% normal goat serum (Sera Lab, Sussex, UK) in phosphate-buffered saline (PBS) for 15 minutes. After 1 hour of incubation with the primary antibody, endogenous peroxidase was blocked for 10 minutes with 0.3% H2O2 in PBS. Subsequently, sections were incubated with biotin-conjugated rabbit antimouse (Dako) for 30 minutes. After incubation with streptavidin-biotin-peroxidase complex (Dako) for 30 minutes, the sections were developed with diaminobenzidine tetrahydrochloride and counterstained with hematoxylin.

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minutes, horseradish peroxidase activity was detected with 3-aminot-9-
ethylcarbazole (Sigma, St Louis, MO) and 0.03% H2O2. Sections were
counterstained with haematoxylin (Merck, Darmstadt, Germany).
RNA isolation and cDNA synthesis. Total cellular RNA was iso-
lated from frozen tissue sections using the TRIZOL reagent (Life
Technologies, Breda, The Netherlands) according to the manufacturer’s
instructions. For cDNA synthesis, 10 μg of RNA was incubated with 5
mmol of pd(N)2 primer (Pharmacia Biotech, Rosendal, The Nether-
lands) for 10 minutes at 65°C. After cooling on ice, the reaction mixture
was added to a final volume of 50 μL. It contained 400 μL of Moloney
murine leukemia virus (M-MLV) reverse transcriptase (Life Technolo-
gies, Breda, The Netherlands), 8 μmol/dishoethanolamine (DTT), 1 mmol/L
de each dNTP, 1× first-strand buffer (50 mmol/L Tris-HCl, pH 8.3, 75
mmol/L KCl, 3 mmol/L MgCl2), and 60 U of RNAsin inhibitor
(Boehringer Mannheim, Almere, The Netherlands). The reaction was
performed for 1 hour at 37°C. Subsequently, the enzyme was inacti-
vated during 10 minutes at 95°C.
Polymerase chain reactions (PCR). The complementarity determin-
ing region 3 (CDR3) was amplified using a forward primer with
specificity for framework region 3 (FR3) in combination with reverse
primers specific for JH (JHseq), Cμ, Cγ (Cγ2), Cα, or C8 (Table 2).
Either 1 μL of the CDNA reaction mixture was used or (for a nested
PCR) 1 μL of PCR product from a VH-family-specific PCR was used.
The PCR mixture contained 1× Taq buffer (20 mmol/L Tris-HCl, 50
mmol/L KCl, pH 8.4), 0.2 mmol/L of each dNTP, 1.5 mmol/L MgCl2,
2 U of Taq polymerase (Life Technologies), and 0.5 μmol/L of each primer. First, 10 cycles of amplification were performed in the thermal
cycler (PTC-100, MJ Research Inc., Watertown, MA), i.e. successively
30 seconds at 95°C, 20 seconds at 57°C, and 20 seconds at 72°C. The
next 40 cycles of amplification consisted of 30 seconds at 95°C, 20
seconds at 55°C, and 20 seconds at 72°C. The reaction was com-
pleted for 6 minutes at 72°C. PCR products were analyzed on a 3% Metaphor
gel (FMC Bioproducts, Rockland, ME). For the VH family-
specific PCR, reactions were performed with one of the VH family-
specific leader primers (Table 2), combined with the appropriate reverse
primer, either JH, Cμ, Cγ, or Cα. The PCR reaction mixture was the
same as for the CDR3-specific PCR, except that 1 U of Taq polymerase
and 0.25 μmol/L of each primer was used. Thirty cycles of 30 seconds at
95°C, 30 seconds at 55°C, and 30 seconds at 72°C were performed. The
reaction was terminated for 6 minutes at 72°C. The PCR products were
analyzed on a 1% standard agarose gel (Sigma).
Cloning and sequencing of PCR products. After excision of the
PCR products from an agarose gel and isolation of DNA with the Qiaex
kit (Qiagen, Hilden, Germany), the PCR products were ligated into
gPEM-T vectors (Promega, Leiden, The Netherlands), according to the
manufacturer’s instructions, and transformed into DH10b bacteria (Life
Technologies). Subsequently, both strands of the inserts were sequenced
by 4 or more colonies to obtain the sequence of the dominant clone,
the consensus sequence. Sequencing was performed with an ABI
sequencer (Perkin Elmer Corp., Norwalk, CT) using the dye-terminator
cycle-sequencing kit (Perkin Elmer Corp.), according to the manufac-
turer’s instructions. To determine the Taq error rate of our experimental
design, 19 clones of CD79a and CD79b were sequenced. These clones
were generated according to the same PCR and cloning procedures as
used for the VH genes. The Taq error frequency thus established is
0.14%, which amounts to 0.4 mutation/VH clone.
Assignment of mutations. The sequences found were compared with
published germline sequences, using the Vbase database5 and DNAPlot
on the Internet (http://www.genetik.uni-koeln.de/dnaplot; programmed
by H.H. Althaus) to identify mutations. Mutations at the last nucleotide
position of the V gene were excluded from the mutational analysis,
because they might result from nucleotide deletions at the joining sites.
To calculate whether the excess or scarcity of replacement mutations in
the FRs had occurred by chance, we used the binomial distribution
model as proposed by Chang and Casali.10 In B cells selected for
antibody expression, there is a counterselection for replacement (R)
mutations in the FR to maintain the structure of the antibody. The ratios
of replacement versus silent (S) mutations in the CDRs are often
higher than expected. However, the R/S values found in the CDRs
cannot be used as arguments for or against antigenic selection. Dönner et
al11 showed that the R/S values of both FR and CDR were higher in

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### Table 1. Description of Cases of PG8CL Analyzed in This Study

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Morphology (type)</th>
<th>Ig isotype</th>
<th>CDR1L*</th>
<th>CDR3</th>
<th>Sex</th>
<th>Age (yr)</th>
<th>Localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Large B-cell lymphoma</td>
<td>IgG</td>
<td>++</td>
<td>-</td>
<td>F</td>
<td>40</td>
<td>Head</td>
</tr>
<tr>
<td>2</td>
<td>Large B-cell lymphoma</td>
<td>Not clear</td>
<td>-</td>
<td>-</td>
<td>M</td>
<td>68</td>
<td>Thorax left</td>
</tr>
<tr>
<td>3</td>
<td>Large B-cell lymphoma</td>
<td>IgM, IgD</td>
<td>-</td>
<td>-</td>
<td>F</td>
<td>66</td>
<td>Left</td>
</tr>
<tr>
<td>4</td>
<td>Large B-cell lymphoma</td>
<td>IgM, IgD</td>
<td>-</td>
<td>-</td>
<td>M</td>
<td>58</td>
<td>Elbow, shoulder</td>
</tr>
<tr>
<td>5</td>
<td>Immunocytoma</td>
<td>IgM, faint IgD</td>
<td>-</td>
<td>+</td>
<td>M</td>
<td>56</td>
<td>Right leg</td>
</tr>
<tr>
<td>6</td>
<td>Immunocytoma</td>
<td>IgM, IgD</td>
<td>-</td>
<td>+</td>
<td>F</td>
<td>74</td>
<td>Chin</td>
</tr>
<tr>
<td>7</td>
<td>Immunocytoma</td>
<td>Not clear</td>
<td>-</td>
<td>-</td>
<td>M</td>
<td>80</td>
<td>Face</td>
</tr>
<tr>
<td>8</td>
<td>Pseudolymphoma</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
<td>F</td>
<td>46</td>
<td>Nose</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not determined.
* +, minimal remnants; ++, nodular networks; ++++, extensive, ill-defined PDC networks.

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### Table 2. Primers Used for VH Gene Amplification

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>VH1</td>
<td>AAAAGCACCAACAGCAGACCTGGACAGG</td>
</tr>
<tr>
<td>VH2A</td>
<td>AAAAGCACCAACAGCAGACCTGGACAGG</td>
</tr>
<tr>
<td>VH2B</td>
<td>AAAAGCACCAACAGCAGACCTGGACAGG</td>
</tr>
<tr>
<td>VH3A</td>
<td>AAAAGCACCAACAGCAGACCTGGACAGG</td>
</tr>
<tr>
<td>VH3B</td>
<td>AAAAGCACCAACAGCAGACCTGGACAGG</td>
</tr>
<tr>
<td>VH4</td>
<td>AAAAGCACCAACAGCAGACCTGGACAGG</td>
</tr>
<tr>
<td>VH5</td>
<td>AAAAGCACCAACAGCAGACCTGGACAGG</td>
</tr>
<tr>
<td>VH6</td>
<td>AAAAGCACCAACAGCAGACCTGGACAGG</td>
</tr>
<tr>
<td>FR3</td>
<td>GAGCCGACATCCCTTTCCCTT</td>
</tr>
<tr>
<td>JH7</td>
<td>GCAGCAAGACAGCTGAAAAAC</td>
</tr>
<tr>
<td>JH8</td>
<td>ATGGAGAGACAGCTGAAAAAC</td>
</tr>
<tr>
<td>Cu</td>
<td>GGGCTGGATCCCTCTG</td>
</tr>
<tr>
<td>Cy</td>
<td>GGGCTGGATCCCTCTG</td>
</tr>
<tr>
<td>Cγ2</td>
<td>GGGCTGGATCCCTCTG</td>
</tr>
<tr>
<td>Cα</td>
<td>GGCACTGAGAAGCTGAAAAAC</td>
</tr>
<tr>
<td>C56</td>
<td>GTGCAGAGAAGCTGAAAAAC</td>
</tr>
</tbody>
</table>

Abbreviation: subscripts indicate the amino acid position of the V gene.
nonproductive and therefore unselected rearrangements than in productive, antigen-selected rearrangements. Also, it can be imagined that, in an already selected Ig with optimal affinity, additional replacement mutations in the CDRs are unfavorable.

RESULTS

Morphology of PCBCLs. The clinical and histological data of the 7 PCBCLs analyzed are summarized in Table 1. The group comprised 4 follicle center cell lymphomas (all diffuse large B-cell lymphomas) and 3 immunocytomas (marginal zone B-cell lymphomas). In addition, a lesion showing the clinical and histologic features of a pseudolymphoma was included in this study (no. 8). Despite the fact that all lymphomas displayed a diffuse growth pattern, areas of FDCs were detected in 5 of the 7 lymphomas (nos. 1, 2, 3, 5, and 6). In PCBCLs no. 5 and 6, extensive ill-defined networks of FDCs were found (Fig 1), which suggests that the FDCs form an integral part of this neoplasm. Interestingly, CD70 expression was also found only in lymphomas no. 5 and 6 (not shown). In the other cases (no. 1, 2, and 3), the FDC clusters were more or less nodular and well circumscribed. Here, it is unclear whether they belong to the tumors or represent the remains of pre-existent reactive follicles that were infiltrated by the neoplastic B cells. These different patterns of FDCs in PCBCL have also been reported by Mori et al.12

Fig 1. Tissue section of lymphoma no. 5 stained for FDCs with antibodies against CD21L (DRC-1). Magnification (A) × 50 and (B) × 125.
Ig isotype expression. The Ig heavy chain isotype of the 7 PCBCls studied was determined immunohistochemically (Table 1) and by PCR, i.e., by amplifying the CDR3 region with an FR3 primer in combination with primers specific for the 5' regions of Cj, Cy (Cy2), Co, or Cb heavy chains (Table 2). Because of differences in length of the diversity (D) genes and random nucleotide additions by terminal deoxynucleotidyltransferase at junctions of the V-D and D-J gene segments, the size of the CDR3 regions varies considerably between B-cell clones.14 Therefore, a CDR3-specific PCR on a polyclonal B-cell population yields products of variable size, visible as multiple bands or a smear pattern after electrophoresis, whereas this PCR on a clonal population yields a single band (Fig 2). In our hands, this CDR3-specific PCR has proven very useful as a screening assay and as a sensitive method to identify the lymphoma-derived Ig when, due to the copresence of nonneoplastic B cells, multiple VH genes are amplified from a tissue specimen. With respect to the assessment of the Ig isotype expression, either immunohistochemically or by PCR, we encountered no discrepancies (compare Tables 1 and 3). For example, in lymphoma no. 6, clear membrane expression of both IgM and IgD was detected by immunohistochemistry (Table 1). As expected, products of single length were amplified by PCR using the FR3 primer in combination with JHseq, Cj, and Cb primers, whereas smears were obtained with Cy2 and Co as downstream primers (Fig 2, left panel). PCR of a pseudolymphoma yielded products of variable lengths in all lanes (Fig 2, right panel). In 4 PCBCls (no. 2, 4, 5, and 7), the two methods to assign the Ig isotype were complementary (Tables 1 and 3); immunostaining of lymphoma no. 5 showed clear membrane expression of IgM and, at most, very weak expression of IgD. However, using PCR, both IgM and IgD were clearly amplified from this lymphoma. Also, in cases nos. 2 and 7, the Ig isotypes used could only be established by PCR. Alternatively, lymphoma no. 4 was found to express both IgM and IgD immunohistochemically, whereas by PCR only IgM expression was found. This was possibly due to the low amount of cDNA available from this lymphoma. In summary, 4 lymphomas (no. 3, 4, 5, and 6) were found to coexpress IgM and IgD, whereas 3 lymphomas (no. 1, 2, and 7) expressed IgG. Interestingly, the CDR3-specific PCR of lymphoma no. 7 yielded single bands in the lanes corresponding to the JHseq, Cj2, and Co primers, which is suggestive of the presence of clonal populations of both IgG and IgA isotypes (Fig 2, middle panel). To analyze whether the IgG and IgA products originated from the same tumor clone, the VH genes were amplified with VH family-specific primers in combination with the constant Cy or Co primers. Subsequent sequencing of the VH-Cy and VH-Co PCR products confirmed that they harbored the same VDJ rearrangement (not shown).

VH, D, and JH gene usage. VH genes were amplified with family-specific VH leader primers (Table 2). On the PCR products thus obtained, we performed a nested CDR3-specific PCR to confirm that a particular VH product originated from the clonal population (data not shown). Subsequently, the VH product was cloned and sequenced. The nucleotide sequences have been deposited at the GenBank database (accession nos. AF052379 through AF052386). The VH sequences were compared with the germline VH genes with the highest homology and, accordingly, the number of somatic mutations was determined (Table 3). Four PCBCls used genes of the VH3 family, whereas VH1, VH4, and VH5 family genes were each found once. Comparison with germile VH gene segments showed that the JH4b gene was present in 4 rearrangements (lymphomas no. 1, 3, 5, and 7). The lymphomas no. 2, 4, and 6 used the JH6c, JH3b, and the JH5b genes, respectively. Corbett et al13 proposed stringent criteria for the assignment of D genes: at least 10 consecutive nucleotides of identity are required to confidently assign a D gene segment. According to these criteria, we could only assign the D21-9 and DXP4 gene segments of lymphomas no. 4 and 6, respectively.

Mutation patterns. All lymphomas expressed extensively mutated VH genes, ranging from 15 to 52, with an average of 28.6 mutations per VH sequence (Table 3). Within our limited set of 7 PCBCls, there was no obvious difference in the number of mutations between IgM"IgD" or IgG"lymphomas. Analysis of the distribution of replacement (R) versus silent (S) mutations demonstrated that 5 of the 7 PCBCls (no. 2, 3, 4, 5, and 6) contained a significantly lower number of R mutations in the FRs (Table 4) than would be expected if mutations had occurred by chance alone, i.e., in the absence of selective forces. Except for lymphoma no. 6, the R/S values within the CDRs were always higher than those within the FRs of the corresponding VH genes.

Intraclonal variation. Information on the intraclonal variation was obtained by sequencing of each lymphoma at least four amplified VH molecules (Table 3 and Fig 3). No differences were found in the sequences of the individual clones of lymphomas no. 1 or 2, which are both IgG-expressing PCBCls. Also, in lymphoma no. 3, which coexpresses IgM and IgD, no intraclonal variation was observed. However, lymphomas no. 4,
VH gene analysis of primary cutaneous B-NHL

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Ig isotype*</th>
<th>VH Family</th>
<th>Closest Germeline Gene</th>
<th>No. of Mutations</th>
<th>% of Homology</th>
<th>D Gene</th>
<th>VH Gene</th>
<th>Intraclonal Variation† (no. of clones sequenced)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>γ</td>
<td>5</td>
<td>V5-61 (CO524)</td>
<td>15</td>
<td>95</td>
<td>NA</td>
<td>JH4b</td>
<td>0.14 (4)</td>
</tr>
<tr>
<td>2</td>
<td>γ</td>
<td>3</td>
<td>V3-7</td>
<td>28</td>
<td>90</td>
<td>NA</td>
<td>JH6c</td>
<td>0.14 (4)</td>
</tr>
<tr>
<td>3</td>
<td>μ, δ</td>
<td>3</td>
<td>V3-23</td>
<td>52</td>
<td>82</td>
<td>NA</td>
<td>JH4b</td>
<td>0.14 (5)</td>
</tr>
<tr>
<td>4</td>
<td>μ</td>
<td>3</td>
<td>V3-7</td>
<td>15</td>
<td>95</td>
<td>D21-9</td>
<td>JH3b</td>
<td>0.6 (8)</td>
</tr>
<tr>
<td>5</td>
<td>μ, δ</td>
<td>3</td>
<td>V3-30 (CO53)</td>
<td>17</td>
<td>94</td>
<td>NA</td>
<td>JH4b</td>
<td>1.4 (5)</td>
</tr>
<tr>
<td>6</td>
<td>μ, δ</td>
<td>4</td>
<td>V4-61 (3d279d)</td>
<td>35</td>
<td>88</td>
<td>DXP4</td>
<td>JH6b</td>
<td>2.6 (5)</td>
</tr>
<tr>
<td>7</td>
<td>γ</td>
<td>1</td>
<td>V1-2 (DP8)</td>
<td>37</td>
<td>87</td>
<td>NA</td>
<td>JH4b</td>
<td>3.5 (5)</td>
</tr>
<tr>
<td>8</td>
<td>γ</td>
<td>1</td>
<td>V1-2 (DP8)</td>
<td>37</td>
<td>87</td>
<td>NA</td>
<td>JH4b</td>
<td>0.2 (5)</td>
</tr>
</tbody>
</table>

Abbreviation: NA, the D gene could not definitely be assigned to a germ line D gene.

*Heavy chain isotype expression as determined by PCR.
†The intraclonal variation is indicated as the number of mutations observed per clone, compared with the consensus sequence.

5, 6, and 7 indicate intraclonal variations of 0.6, 1.4, 2.6, and 3.4 mutations/clone, respectively. Moreover, in each of these lymphomas, mutations were found that were shared by more than one clone (Fig 3), which is strong evidence for ongoing somatic hypermutation rather than Taq error. Lymphomas no. 4, 5, and 6 coexpressed IgM and IgD (Tables 1 and 3), as determined by immunohistochemistry and PCR. The highest level of intraclonal variation was observed in the IgG clone of lymphoma no. 7, demonstrating that ongoing mutation is not necessarily confined to IgM* PCBCls. However, in contrast to the high mutation frequency of the IgG* tumor cells, the intraclonal variation found in the IgA* tumor cells amounted to 0.2 mutations/clone, which does not exceed the Taq error frequency of 0.14% (~0.4 mutation/clone). Yet, the consensus sequences of the IgG- and the IgA-expressing subclones were identical.

**DISCUSSION**

In this study, the VH genes of 7 PCBCls were analyzed to learn what mutation steps were traversed by the tumor cells and to gain insight into the biological relation with other B-NHL. The PCR method used here has proven to be a fast and reliable approach to establish clonality at molecular level.
Fig 3. Intraclass variation found in lymphomas no. 4, 5, and 6 and the IgG-clone of no. 7. Indicated are only the codons in which mutations were found. The first clone of each lymphoma is the consensus sequence. The mutations compared with this consensus sequence are shown. Replacement mutations are shown in capitals, and silent mutations are shown in small letters.

esized that these cells represent recent immigrants that are in the process of forming a germinal center. In accordance with this finding, a subset of IgM^IgD^ germinal center B cells has also been noticed by others, carrying, on average, 5.8 somatic mutations per VH gene.7 A minority of these CD38^IgM^IgD^ B cells harbored more than 10 mutations in the expressed VH genes. Because the patterns of somatic mutations in these cells were suggestive of antigen-driven selection processes, the investigators hypothesized that they might represent either germinal center founder cells derived from recirculating IgM^IgD^ memory cells or centrocytes differentiating into IgM^IgD^ memory B cells.5

The presence of somatic mutations in the VH genes indicates that PCBCls may be derived from germinal center cells or their descendants. Accordingly, in 5 of the 7 PCBCls studied (no. 2, 3, 4, 5, and 6), the FRs displayed R/S ratios that are significantly lower than would be expected if random mutation would have occurred in the absence of selective forces. The apparent counterselection against R mutations in the FRs, which are essential for the integrity of the antibody, implies that expression of proper antigen receptors has been important for cell survival, at least at some stage(s) of development. However, the fact that the mutation patterns in these PCBCls are reminiscent of antigen selection does not necessarily signify that lymphoma-genesis itself is antigen-driven. In the diffuse large-cell lymphomas no. 1, 2, and 3, no intraclass variation was observed. In these lymphomas, the somatic mutations were most likely introduced before or at the moment of complete transformation; therefore, it cannot be concluded that these lymphomas need Ig expression for their survival, let alone antigen recognition. The fact that the mutation frequency in these cases is abnormally high may be the result of a prolonged stay in the germinal center environment, possibly due to early, pretransforming genetic alterations. The findings within these PCBCls are in accordance with a recent report by Gellrich et al.29 who analyzed, using single-cell PCR, the VH gene of a primary cutaneous immunoblastic B-cell lymphoma of the leg. It was demonstrated that the VH gene expressed by this lymphoma harbored 39 nucleotide differences compared with the most homologous VH gene (DP-54/V3-7). No evidence was obtained for ongoing somatic hypermutation. This subset of large-cell PCBL, with a high load of somatic mutations without ongoing mutation,
resembles noncutaneous diffuse large B-cell lymphomas. However, our data do not provide an explanation for the difference in clinical behavior between cutaneous and extracutaneous diffuse large B-cell lymphomas.

Interestingly, in the PCBCLS no. 4, 5, 6, and 7, intrachromal variation was found, indicative of ongoing somatic hypermutation. The degree of intrachromal variation ranged between 0.6 and 3.4 mutations per clone. In lymphoma no. 7, clonal IgG and IgA gene products were found that contained the same VDJ rearrangement, indicating that they were isotype switch variants of the same tumor. It is remarkable that, whereas a high level of intrachromal variation was observed in the IgG clone (3.4 mutations/clone; Table 4), the IgA clone displayed no significant intrachromal variation. Still, the consensus sequences of the IgG-expressing and IgA-expressing clones proved to be exactly the same. The latter finding may indicate that the Ig heavy chain isotype switching occurred relatively late after the moment of transformation. The absence of somatic hypermutation in the IgA subset demonstrates that the ability to mutate may be abolished in the course of disease. This finding may have several explanations. It can be reasoned that the IgG clones were already functionally heterogeneous and that the isotype switch to IgA occurred in a nonmutating subclone. Alternatively, the shut-off of the mutation machinery may somehow have coincided with the process of isotype switching. It has been demonstrated that, in normal B cells, heavy chain class switch does not per se terminate somatic mutation. It is possible that additional genetic damage caused the termination of somatic hypermutation.

The VH mutation patterns of PCBCLS no. 4, 5, and 6 are also suggestive of clonal selection processes that favor BCR expression. In this subset of PCBCLS, the somatic mutations found may have been introduced both before and, contrary to their nonmutating counterparts, after the moment of complete transformation. It can be assumed that, among the mutations introduced in the lymphoma cells, there will be those that give rise to stopcodons or nonfunctional frameshifts. In fact, because the Ig genes are single-copy genes, they are particularly prone to inactivation by such mutations. Thus, the finding that actively mutating lymphomas express Ig is not trivial and implies that the tumorigenesis of these neoplasms may be BCR-guided. In this respect, the finding that two of four mutating PCBCLS (no. 5 and 6) contain elaborate networks of FDCs is noteworthy. Because normal skin does not harbor FDCs, this suggests that the FDCs may be an essential part of these neoplasms and possibly have a role in tumorigenesis. This idea is supported by the strong CD70 expression found on these particular PCBCLS. CD70 has been described to be a marker for mature B cells that have recently been primed by antigen in vivo. On the other hand, 2 PCBCLS that actively mutate their VH genes are devoid of FDCs (no. 4 and 7). Assuming that this is not due to sampling error, this would signify that somatic hypermutation in PCBCLS does not necessarily depend on the presence of FDCs.

In line with the presumed analogy of the tertiary lymphoid tissue of skin (skin-associated lymphoid tissue [SALT]) and mucosa (MALT), some investigators propagate the idea that PCBCLS may be biologically related to malignant lymphomas of MALT. On morphological grounds, this has particularly been suggested for the immunocytomas of the skin. Although preliminary, our data provide support for this concept. Of the noncutaneous B-NHLs, ongoing somatic hypermutation is a characteristic feature of MALT lymphomas and follicular lymphomas. A potential relationship between PCBCLS and follicular lymphomas is supported by the presence of FDCs in the majority of the PCBCLS. Although our data suggest a relationship between PCBCLS and various forms of noncutaneous B-NHLs, at this moment no definite conclusions are allowed. Additional molecular data on the genetic defects have to be awaited to clarify this relationship.

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