Hairy cell leukemia (HCL) commonly expresses multiple immunoglobulin isotypes, a feature rare in other B-cell malignancies or in normal B cells. In HCL, there is no phenotypic evidence for subpopulations, and single cells from one previous case contained transcripts for several isotypes. This raises the questions of the differentiation status of the cell of origin and of posttransformation events. We have investigated 9 cases, all expressing multiple immunoglobulin isotypes. Multiple tumor–derived variable–(diversity)–joining–constant μ, δ, γ, α (V(D)J–Cμ, δ, γ, α) transcripts were confirmed in single cells of a further case. All cases were negative for germinal center (GC)–associated markers CD27 and CD38. Seven of 9 cases had mutated VH genes, with low levels of intraclonal heterogeneity, but 2 of 9 were unmaturated, indicative of pre-GC origin. Eight of 9 cases expressed activation-induced cytidine deaminase (AID), a molecule essential for somatic mutation and isotype switch. All cases expressed germ line heavy-chain I exon (Iγ–Cγ) transcripts which paralleled surface immunoglobulin (sIg) isotype. Significantly, no circle transcripts indicative of deletional recombination of switched isotypes were detectable in 9 of 9 cases. These data indicate heterogeneity in the cell of origin in terms of mutational status, but reveal common features of AID expression and isotype-switching events occurring prior to deletional recombination. Both mutational and switching events may be influenced by environmental factors at extrafollicular sites. (Blood. 2004;104: 3312-3317) © 2004 by The American Society of Hematology

Introduction

Hairy cell leukemia (HCL) is a rare, chronic B-cell disorder characterized by leukemic hairy cells (HCs) present in bone, blood, marrow, and splenic red pulp, with atrophy of white pulp in some cases. Lymph node involvement is infrequent. HCL is typically associated with markers of activation that include expression of CD25, CD11c, FMC7, and CD103 at high intensity.1 A distinctive feature is that approximately 40% of HCLs express multiple surface immunoglobulin (sIg) isotypes.1

Immunoglobulin variable (V) region gene analysis delineates critical features of the clonal history of the cell of origin.2 It identifies whether antigen encounter by a normal mature B cell has activated somatic mutation. This is generally restricted to the germinal center (GC), and isotype-switching events may also occur there.3 For both mechanisms, the enzyme activation-induced cytidine deaminase (AID) is critical.4 The precise role of AID-mediated deamination of cytidine in the mutasome or switch recombinase is becoming clearer.5 Domains in AID regulating somatic mutation or isotype switching have now been mapped, with point mutations in the C-terminus ablating switch.6,7 AID is normally expressed in GC B cells, mainly at the CD19+CD38+CD44+ centroblast stage,8 and is inducible with CD40 ligand (CD40L) and cytokine signals, with Pax-5 transcriptionally active.9,10 Although these processes commonly take place in the GC, this site is not an absolute requirement for mutational and switching events.8

AID is constitutively expressed in a large proportion of, but not all, GC-derived B-cell tumors, such as follicular lymphoma, diffuse large B-cell lymphoma, and Burkitt lymphoma.8,11 AID is absent in some pre-GC and post-GC tumors, such as lymphoblastic leukemia and multiple myeloma.8,11 However, B-cell tumor categories can display subsets with mutated or unmutated VH genes, such as chronic lymphocytic leukemia (CLL) and mantle cell lymphoma (MCL), indicative of pre- or post-GC origins.12-15 In these subsets, AID expression does not associate with accumulation of somatic mutations in VH genes and often parallels ongoing switching events, most likely occurring in tumor subpopulations.12,13,15 To date, AID expression does not appear to have been investigated in HCL.

HCL was previously shown to display mainly somatically mutated VH genes, and this appeared to be a general feature, found in cases expressing single or multiple sIg isotypes, together with a low degree of intraclonal heterogeneity.16,17 In the significant proportion of cases expressing multiple sIg isotypes, we reported that isotypic variant transcripts are expressed and, in the one case investigated, were coexpressed in individual cells.17 This surprising observation suggested that HCs arrest during isotype switching at a stage in which RNA processing precedes deletional recombination.17 We have probed this question further in more cases and have used immunogenetic and phenotypic analysis to investigate the point of differentiation reached by this subset of HCLs. Our data confirm the presence of multiple isotypes in single cells of a further

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An Inside Blood analysis of this article appears in the front of this issue.

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available case. Somatic mutational status was heterogeneous, with most cases undergoing somatic mutation, but a minority remaining unmutated. The phenotypic analysis and lack of switch circles support the concept that these tumors are undergoing a nondele-
tional isotype-switching process. Although HCL appears not to be GC associated, AID is induced in the majority of cases, even when unmutated, possibly in anticipation of subsequent deletional recombination.

Patients, materials, and methods

Patients

Nine unselected HCL cases, referred to the Division of Hematology in the University of Siena (Siena, Italy), were investigated. Diagnosis of HCL was based on immunohistochemical examination of bone marrow biopsies and morphologic and immunophenotypic identification of HCs in the peripheral blood.1,17 Three cases (nos. 42, 83, and 93) were previously shown to express multiple isotype transcripts.17

Phenotypic analysis

Immunophenotypic studies on HCs, including surface expression of immunoglobulin M (IgM), IgD, IgG, and IgA and germinal center markers CD27 and CD38, were carried out as previously described.17 In 2 cases (nos. 83 and 93) where additional material was available, immunoglobulin isotype expression was also performed by a dual isotype–staining techni-
qure. As in the previous analysis, HCs were first incubated overnight in RPMI medium with 10% fetal calf serum and fractionated again. HCs were then incubated with peridinin chlorophyll-alpha–protein–anti-CD20 (PerCP–anti-CD20) (BD Pharmingen, San Diego, CA); phycoerythrin–F(ab')2 anti-IgD or FITC–F(ab')2 anti-IgG (Caltag Laboratories, Burlin-
game, CA). Negative control was performed by incubating cells with PerCP–anti-CD20 and appropriate fluorochrome-conjugated isotype controls for each anti-isotypic antibody. HCs were identified as high side scatter (SSC) and high forward scatter (FSC) CD20<sup>hi</sup> cells.18 Expression of each immunoglobulin isotype was designated as positive when the shift of test histogram was at least 1.5 × that of the isotype control.

Identification of tumor-derived VH genes

Tumor-derived VH transcripts were identified and mutation patterns ana-
lyzed as reported previously.17 Briefly, RNA was isolated from mono-
nuclear cells and reverse transcribed to cDNA with the use of oligodeoxy-
thymidine (oligo-d(T)). One fifth of the cDNA was subjected to amplification. Tumor VH transcripts were amplified with the use of a mixture of 5<sup>V</sup>-VH leader primers with downstream 3′ primers specific for each expressed constant region.17 Amplified products were cloned as described, and individual plasmids from different clones sequenced with the use of an automated in-house core facility.17 The data were analyzed by means of MacVector 4.5.3 software (Accelrys, San Diego, CA) and aligned to Entrez and V-BASE databases.19,20 Transcripts were considered tumor derived when they shared the same CDR3 sequence. Intrachromosomal heterogeneity was assessed in the cloned products and was distinguished from Taq fidelity by an increased frequency as compared with the Taq error rate and by the finding of the same mutations in more than one clone and/or in different polymerase chain reactions (PCRs). Qiagen (Valencia, CA) Taq polymerase error rate was assessed as previously described.17

Analysis of multiple tumor-derived V(D)J switch transcripts in single hairy cells

Material from case 266 was available for further analysis. The protocol established previously for identifying tumor-derived switch-variant tran-
scripts in single HCs was used.17 Briefly, leukemic cells prelabeled with CD11c were identified as high SSC, high FSC peripheral blood mono-
nuclear cells (PBMCs), and CD11c<sup>-</sup> for sorting as single cells by means of

an automated cell-deposition FACS Vantage instrument (Becton Dickinson, San Jose, CA). Single cells were sorted directly into buffer for cDNA synthesis, and individual IgM/IgD/IgG/IgA transcripts identified by semi-
nested PCR amplification with a respective downstream C<sub>M</sub>, C<sub>B</sub>, C<sub>y</sub>, C<sub>a</sub> primer as reported.17 Amplified DNA of expected size was purified from agarose gels and directly sequenced bidirectionally.

Expression of AID

AID expression was determined by reverse transcription–PCR (RT-PCR) as described.15,21 Briefly, 30 amplification cycles were carried out with the use of the forward 5′-ATGGACAGGCTCTTGTGAAAC-3′ and reverse 5′-CTCAGAACATCAACCTCATACA-3′ primers and an annealing tem-
perature of 50°C. These primers distinguish wild-type and splice variants of AID on the basis of size of the amplified product. Amplified AID and variant transcripts were cloned for sequence analysis, as described previously.15 Results were confirmed by replicate RT-PCR.

Investigation of sterile germ line switch transcripts

Germ line heavy-chain I exon (I<sub>5</sub>-C<sub>M</sub>-C<sub>B</sub>) transcripts were evaluated with the use of the methodology described by Cerutti et al.22 Briefly, Ig-<i>C</i>-Cy and I<sub>0</sub>-Cy transcripts were identified by RT-PCR amplification using 30 cycles and consensus I<sub>y</sub>, I<sub>x</sub>, and I<sub>y</sub>, I<sub>x</sub>, C<sub>y</sub> primers as reported.22 Amplified DNA of predicted size was cloned for sequence analysis to verify identity of germ line transcripts.

Investigation of switch circle transcripts

I<sub>y</sub>-C<sub>M</sub>, I<sub>x</sub>-Cy<sub>x</sub>, and I<sub>y</sub>-Cy<sub>y</sub> circle transcripts were sought by means of RT-PCR. Primers and conditions for each amplification were established elsewhere.22,23 Briefly, 3 μL cDNA from each case was subjected to amplification with the use of the reverse C<sub>y</sub> primer 5′-GTGGCGGGTT-
GGGTTGCTGAC-3′ together with either the forward I<sub>y</sub> 5′-GCGTCC-
CAAGCAACAGGGCAGGACA-3′ primer (for I<sub>y</sub>-Cy<sub>x</sub> circle transcript) or the forward I<sub>x</sub> 5′-CAGCAGCCCTTGTGGCAGGCCGAC-3′ primer (for I<sub>y</sub>-Cy<sub>y</sub> circle transcript). Amplification of I<sub>x</sub>-Cy<sub>x</sub> circle transcript was carried out with the reverse Cy primer 5′-CAAGCTGCTG-
GAAGGAGCAAGGT-3′ and the forward I<sub>y</sub> primer. All RT-PCR amplifi-
cations were performed with an annealing temperature of 68°C for 30 cycles.

Use of primers and amplification conditions to identify switch circle transcripts was validated with the use of control cDNA from 3 CLL cases with unmutated VH<sub>1</sub> genes, as reported.15

Results

Multiple isotype expression in HCL

All 9 HCL cases expressed multiple sIg isotypes, with IgM<sup>+</sup>IgD<sup>+</sup> or IgD<sup>+</sup>HCs expressing IgG with or without IgA (Table 1). In each case, the expressed isotypes were found on the large majority of HCs, with no evidence for subpopulations (data not shown). To further corroborate our observation of multiple isotypes expressed on single tumor cells, we performed dual isotype staining of HCs from 2 cases (83 and 93). In this experiment, HCs were identified as high (high) FSC/hiSSC PBMCs, CD20<sup>high</sup>+ events.18 Results are shown in Figure 1. Clearly, in both cases the great majority of the HCs coexpressed IgM/IgD and, more interestingly, IgM/IgG proteins. In particular, in case 83, 96% and 93% of the HCs showed concomitant surface expression of IgM/IgD or IgM/IgG proteins, respectively. In case 93, IgM/IgD or IgM/IgG surface coexpression was observed in 91% and 95% of the HCs, respectively. In our analysis of multiple sIg expression, any nonspecific reactivity of antibodies used was excluded by repeating the analysis in mantle cell lymphoma cases, where neoplastic cells from the peripheral blood showed surface expression of IgM and IgD, but not of IgG.
Table 1. Immunogenetic and phenotypic features in HCL

<table>
<thead>
<tr>
<th>Case</th>
<th>IgM</th>
<th>IgD</th>
<th>IgG</th>
<th>IgA</th>
<th>V_H</th>
<th>% homology†</th>
<th>J_H</th>
<th>D</th>
<th>No. tumor-derived clones/total no. clones</th>
<th>Intrachain heterogeneity‡</th>
<th>AID</th>
<th>CD27</th>
<th>CD38</th>
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<td>+</td>
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<td>−</td>
<td>3-33</td>
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<td>(wt)</td>
<td>(Ex4)</td>
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<tr>
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<td>+</td>
<td>−</td>
<td>−</td>
<td>3-23</td>
<td>97.6</td>
<td>4b</td>
<td>2-21</td>
<td>8/8</td>
<td>+</td>
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<td>(wt)</td>
<td>(Ex4)</td>
</tr>
<tr>
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<td>+</td>
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<td>−</td>
<td>3-33</td>
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<td>6b</td>
<td>4-11</td>
<td>7/8</td>
<td>+</td>
<td>+</td>
<td>(wt)</td>
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<td>−</td>
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<td>2</td>
<td>1-26</td>
<td>4/4</td>
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<td>(wt)</td>
<td>(Ex4)</td>
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<td>+</td>
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<td>100</td>
<td>4b</td>
<td>6-19</td>
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<td>9/9</td>
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<td>+</td>
<td>(wt)</td>
<td>(Ex4)</td>
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<td>−</td>
<td>3-30</td>
<td>100</td>
<td>4b</td>
<td>4-17</td>
<td>10/10</td>
<td>−</td>
<td>+</td>
<td>(wt)</td>
<td>(Ex4)</td>
</tr>
</tbody>
</table>

†(wt) indicates wild-type AID transcript identified as a 570 bp band after amplification by RT-PCR. ‡(Ex4), identification of AID splice variant transcript with loss of exon 4 (454 bp).

*Expression for each isotype was defined as positive (+) when mean fluorescence intensity was higher than 1.5. Expression of each isotype was determined on the CD19/CD11c HCs. Within the HCs, there was no evidence for subpopulations, with the majority of tumor events expressing each isotype.

‡Intraclonal heterogeneity was defined as previously described. VH gene data on cases 42, 83, and 93 were reported previously.

*Expression for each isotype was defined as positive (+) when mean fluorescence intensity was higher than 1.5. Expression of each isotype was determined on the CD19/CD11c HCs. Within the HCs, there was no evidence for subpopulations, with the majority of tumor events expressing each isotype.

HCs do not express the GC-related CD38 and CD27 markers

Each of the HCL cases had a typical activated phenotype, with expression of CD25, CD103, CD11c, FMC7, slg, CD20, and CD79b at high intensity. Notably, in 9 of 9 HCL cases, CD38 and CD27 expression were not detected (Table 1).

Analysis of tumor V_H genes

Table 1 shows the number of tumor-derived clones, as identified by a common CDR3 signature sequence among multiple immunoglobulin clones amplified from each patient, together with tumor V_H, D, and J_H gene segment usage and deviation in homology from the germ line counterpart. Nucleotide sequences of each case have been deposited in the GenBank databases (accession nos. AF302821-23 and AY345917-22; http://www.ncbi.nlm.nih.gov/genbank/index.html). V_H gene segments belonged to the V_H3 family in 7 of 9 cases while the remaining 2 belonged to the V_H4 and V_H1 families. J_H4b gene segment was the most commonly used J_H segment (6 of 9 cases).

Somatic mutations were revealed in 7 of 9 cases (91.4%-97.6% homology to germ line). In 6 of the 7 cases deriving from a mutated cell of origin, we were able to confirm the presence of a low level of intrachain heterogeneity (Table 1). This is consistent with ongoing mutational events targeting the V_H locus. Intrachain variation was not documented in case E70, probably owing to the few clones (4) analyzed.

Completely unmutated sequences were observed in 2 of 9 cases (Table 1). Both unmutated cases used the V_3-30 and J_H4b gene segments. A minority of HCL cases carrying V_H genes with greater than 98% homology to germ line have been previously reported. While greater than 98% homology has been a convenient assignment criterion for “unmutated” CLL, it is possible that the low number of base changes within the 2% deviation from germ line sequence can represent real mutations.

We have evidence for this in some cases of HCL. However, in cases G13 and 283 with germ line V_H genes and isotype-switching variants, no mutations were detectable at the nucleotide level, indicating a new minor subset in multiple sIg-expressing HCL with no activation of the mutational machinery. It is as yet not clear whether this minor somatically unmutated subset also exists in the approximately 60% of HCLs with single-slg expression. In a recent preliminary report, 6 of 34 cases of HCL were found to be unmutated, but as yet the criteria used to define unmutated status or the nature of slg expression in these cases have not been available.

Importantly, we confirmed our previous observation that multiple tumor-derived mature isotype-variant transcripts are expressed in single HCs. In case 266, multiple V(D)J-C_\(\mu\), 6, 4, \(\alpha\), and IgA (data not shown). These results further reinforce our previous observations that HCs express multiple isoforms with no evidence of subpopulations, at both the protein and the transcript levels, the latter previously documented in 3 cases from this series and extended in a further 3 cases in this study (Table 2).
transcripts were simultaneously identified in 7 of 10 single isolated cells (Table 3). In this case, expression of tumor C8 transcripts did not yield measurable sIgA expression (Table 1).

**AID is expressed in HCL**

AID transcript expression was sought by qualitative RT-PCR. With our primers and conditions, AID cannot be detected from PBMCs of healthy individuals even when the reaction is carried out for 40 cycles (data not shown). In HCL, with a 30-cycle reaction, AID was found to be expressed in 8 of 9 cases (Table 1). Expression paralleled ongoing mutation in 5 of 6 cases, as expected. However, AID was also expressed in the 2 unmutated cases (Table 1). Sequence analysis of AID transcripts revealed no mutations, indicating potential functionality. In both subsets, a variant AID transcript was frequently identified in addition to the wild-type transcript (Table 1). The functional significance of the variant transcript is as yet unclear, although with loss of exon 4, it is unlikely to play a role in switch events.

**HCLs reveal germ line switch transcripts but absence of circle transcripts**

Early switch events in these HCLs were investigated, revealing initiation of isotype-switching activity in each case by detection of Iγ-Cγ (9 of 9 cases) and Iα-Cα (1 of 9 cases) germ line transcripts (Figure 2). Expression of multiple germ line transcripts were observed in several individual cases; of note is case 93, where 3 distinct Iv1-3 subclass transcripts were apparent from sequence analysis (Table 2). These germ line transcripts paralleled expression of surface immunoglobulin isotype, notably in case 216, where Iα-Cα and Iγ-Cγ were coexpressed with sIgG/sIgA (Table 1). They also generally correspond to the isotype profile of the V(D)J-Cβ transcripts (Table 2). Contamination of germ line transcripts from nontumor lymphocytes was excluded by analysis of cDNA from 3 healthy peripheral B lymphocytes, which were all negative when 30 or 40 PCR cycles were used (Figure 2). To further define switch events, we sought circle transcripts. Their detection would identify the stage at which DNA deletional recombination is occurring. However, circle transcripts could not be detected in any of our HCL cases, under assay conditions in which 3 CLL cases were found positive for Iγ-Cγ and Iα-Cα circle transcripts (Figure 3). Lack of circle transcripts in our HCLs expressing multiple isotypes is concordant with their stage of arrest, prior to deletional recombination.

**Discussion**

B-cell tumors arrest at different points of normal B-cell differentiation and tend to conserve phenotypic and immunogenetic features of the B cell of origin. It is possible to deduce from the analysis of the V genes whether the cell of origin has undergone the processes of somatic mutation and isotype switching and whether there are continuing influences driving these processes, even in the arrested cell. Often this analysis will reveal subsets within previous classifications, some of which have significant clinical importance. It has been difficult to classify the differentiation status of HCL, and the presence of multiple immunoglobulin isotypes has presented a particular problem.

Several B-cell tumors can express dual isotypes, and the assumption has been that there are subpopulations within the clone, some of which have undergone deletional isotype switching. These events are initiated by signals that activate transcription of the Il4 exons, located 5’ of individual switch (S) regions, and transcripts are then spliced to generate known germ line transcripts. These germ line transcripts are generated for each CH isotype or subclass as selected, and only those Il4 exons upstream of the CH regions that are being targeted for switch recombination are transcribed. Isotype-switching recombination then proceeds to

---

**Table 3. Alternative isotype transcripts in single-cell hairy cells from patient 266**

<table>
<thead>
<tr>
<th>Cell</th>
<th>IgM</th>
<th>IgD</th>
<th>IgG</th>
<th>IgA</th>
</tr>
</thead>
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<tr>
<td>1</td>
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<td>—</td>
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<tr>
<td>10</td>
<td>—</td>
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</tr>
</tbody>
</table>

* indicates that isotype transcripts were detected; —, that isotype transcripts were not detected

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**Figure 2. Analysis of sterile germ line switch transcripts in HCL expressing multiple sIg isotypes.** Germline transcripts are transcribed from 1 exon promoters during initiation of isotype-switching events, and splicing generates Iγ-CH and Iα-CH transcripts. Germline transcripts were sought in our HCL cases by using RT-PCR employing primers and assay conditions as reported. Both Iα-CH and Iγ-CH transcripts of predicted size, as indicated, were identified and paralleled sIg expression in each case. Each RT-PCR product was excised and sequenced to verify the identity of transcripts. Minor bands in a few cases were shown by sequence analysis to be unrelated nonspecific amplification products. To exclude contamination from normal circulating lymphocytes, germ line transcripts were also assayed in cDNA generated from 3 healthy peripheral lymphocyte control preparations (labeled A, B, or C), and these were found to be negative even when 40 PCR cycles were used. Template cDNA levels were compared by reference to V4 (V) or β-actin (β) genes as shown.

**Figure 3. Analysis of switch circle transcripts in HCL expressing multiple sIg isotypes.** Circle transcripts are a hallmark of active class switching deletional recombination, and these were analyzed in our series of HCL cases by an RT-PCR method as described previously. All HCL cases were found negative for both Iα-Cα and Iγ-Cγ circle transcripts as shown. Template cDNA input was compared by using identical amounts of cDNA for amplification of V1 as a reference gene (V). Assay conditions were validated by identifying CTs, confirmed by sequence analysis, in comparable amounts of cDNA from 3 control cases of CLL displaying unmutated V1 genes (C1, C2, C3), which exhibit ongoing switching deletional events as reported. For CLL, β-actin was used as a loading marker gene (labeled B).
take place between 2 switch (S) regions located 5' of each C\(_{H}\) gene. The intervening DNA segments are looped out by deletional recombination, resulting in extrachromosomal circular DNA (switch circle). The switch circle includes the \(I_{g}\) promoter upstream of the targeted S region and \(C_{\mu}\), and is able to transcribe an \(I_{g}\)-\(C_{\mu}\) "circle transcript." In B cells undergoing isotype switch, therefore, detection of circle transcripts verifies that deletional events are occurring. This is the case for the unmutated subset of CLL, where transcripts generated by ongoing switching events have been identified, and which were used as a control for our study.

In HCL, phenotypic evidence points to the expression of multiple isotypes by each cell, rather than to the presence of subpopulations that have undergone deletional switch. While, owing to possible binding to Fc receptors, the use of whole IgG antibodies for detection of expressed immunoglobulin classes could be faulted, F(ab')\(_2\) reagents have confirmed this finding. We reproduced this on our cases, and corroborated findings by the amplification of multiple transcripts from separated single tumor cells in one case. This finding substantiated our previous observations of multiple variant isotypes in single HCs. As a further consequence of these underlying events, we failed to detect deleted circle transcripts, even when such transcripts were readily identifiable in cases of CLL, where transcripts generated by ongoing switching events have been identified, and which were used as a control for our study.

This finding substantiated our previous observations of multiple variant isotypes in single HCs. As a further consequence of these underlying events, we failed to detect deleted circle transcripts, even when such transcripts were readily identifiable in cases of CLL. The weight of evidence points to arrest at a point prior to deletional switch but where multiple isotypes can be generated. The mechanism could be RNA processing, either from a long transcript or via trans-spliced chimeric germ line transcripts, proposed to act as "bridging templates" for normal isotype switching. Another possibility is a mechanism whereby the V(D)J locus is duplicated and inserted into downstream switch region(s), and this has been mapped in a cell line that spontaneously switches at a high rate. The duplicated V(D)J gene is inserted into the S\(Y_{1}\) site, leading to loss of the S\(Y_{1}\) exon. Consequently, sterile S\(Y_{1}\) transcripts would not be expected. These V(D)J duplication and insertional events are rare in B-cell tumors, and have been implicated in only one dual isotype–expressing CLL case to date.

This mechanism could account for multiple isotype expression in single cells, but in cells expressing IgM, IgG, and IgA, V(D)J insertion events upstream of \(C_{\gamma}\) and \(C_{\alpha}\) would be required. This seems unlikely given the low frequency of such events. Another argument against this mechanism is that insertions would silence production of sterile transcripts as S\(Y_{1}\) exons are deleted. However, transcripts, often derived from more than one isotype or IgG subclass, were found in all cases. Taken together with the absence of circle transcripts, the generation of the multiple transcripts can be most likely explained as occurring at the RNA level, by a mechanism not yet defined.

Sterile germ line switch transcripts are further indicative of functional upstream switching signals. These germ line transcripts precede AID expression, as they can be identified in AID\(^{-/}\) B cells. Recently however, it has been shown that germ line transcription plays a dominant role in controlling locus accessibility of AID in class switch recombination, which then associates with an RNA polymerase II complex at switch sites. Interestingly, in HCL, chromatin accessibility for AID to initiate class switch recombination is clearly feasible owing to germ line transcripts, suggesting that other factors underlie arrest prior to deletion. Clearly this point of arrest applies only to the approximately 40% of cases of HCL that express multiple isotypes. Other cases of HCL, which express a single immunoglobulin isotype, appear to have achieved deletional isotype switch. However, it does raise the question of whether it represents an aberrant pathway or reflects a transient point of differentiation traversed by normal B cells.

As for many B-cell tumors previously classified as a single entity, HCL includes subsets, reflecting transformation across a range of points of differentiation. Unifying immunohistochemical features, now including the lack of CD27 or CD38 expression, indicate that these are not GC tumors. Both CD38 and CD27 are GC markers in normal B cells, associating with the centrocyte and centroblast stages, with CD27 being retained by memory B cells. These features suggest that in HCL, ongoing somatic mutation in V genes is occurring independently of a GC site. Isotype-switching events are common and also occur at these sites. Somatic mutations in V genes can occur in the absence of GCs. In patients genetically deficient in CD40 ligand, somatic mutation can also occur through alternative pathways. In a human lymphoma cell line, engagement of the B-cell receptor can mediate somatic mutation in the absence of activated T-cells. Similarly, isotype switching can also occur at non-GC sites. HCs show no histologic or phenotypic evidence for interactions with a conventional GC and may be activated by similar processes. Recent gene expression data in HCL appear consistent with a post-GC B-cell profile. In that study, HCL cases lacked expression of genes encoding the specific GC markers CD10, BCL-6, and CD38. Levels of CD27 expression, assayed as cRNA transcripts, were heterogeneous and lower than in memory B cells and included some HCL cases that were negative. In our series, both CD38 and CD27 were absent at the protein level in HCL cells, and this has been further confirmed for a total of 17 of 17 cases from our clinic (F.F., D.R., F.L., unpublished data, March 2004).

Taken together, these data strongly suggest activation of hairy leukemic cells at non-GC sites, arrested at the stage of somatic mutation and isotype-switching events and still subject to environmental stimuli. Whether the features of HCL represent disordered cellular machinery or are the consequence of occupation of an unusual site remains to be assessed. However, as always, tumors may be revealing unexpected aspects of normal B-cell behavior.

References


Hairy cell leukemia: at the crossroad of somatic mutation and isotype switch

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