Chronic lymphocytic leukemia preceded by cold agglutinin disease: intrachonal immunoglobulin light-chain diversity in \( V_{H}4-34 \) expressing single leukemic B cells

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Autoimmune phenomena may precede or accompany lymphoid malignancies, especially B-chronic lymphocytic leukemia (B-CLL). We report a patient with a 7-year history of primary (idiopathic) cold agglutinin (CA) disease whom B-CLL subsequently developed. Immunophenotyping and single-cell reverse transcription–polymerase chain reaction (RT-PCR) were applied to investigate the origin and diversification of leukemic B cells. The obtained data indicate a memory cell-type origin of the B-CLL cells. Remarkably, the \( IgV_{H} \) genes of the B-CLL cells showed intrachonal diversity, whereas the mutational pattern of their paired \( IgV_{\mu} \) genes were invariant. Thus, the light-chain–restricted intrachonal diversity in individual leukemic B cells in this patient strongly indicates a differential regulation or selection of the ongoing mutational process. Of note, our findings suggest that this B-CLL had developed from the patient’s CA-producing B-cell population.

**Introduction**

Several humoral autoimmune phenomena have been reported in B-chronic lymphocytic leukemia (B-CLL), including autoimmune hemolytic anemia. However, it remains uncertain whether these phenomena arise from the malignant cells or from residual B cells. In both possibilities, autoimmunity should develop in association with or following malignancy. On the other hand, autoimmune dysregulation is a well-known risk factor of the development of lymphoid malignancies. B-CLL developed in a patient with existing chronic B-cell dyscrasia that had become clinically apparent as primary cold agglutinin disease (CAD), which is generally regarded as premalignant or low-grade malignant lymphoproliferation. Thus, a B-CLL origin from the cold agglutinin (CA)–expressing population was likely. Because the respective autoantigens in CAD, mostly belonging to the I/i system, are widely expressed on healthy red blood cells, high antigenic pressure on the proliferating B cells may be hypothesized. Using single-cell reverse transcription–polymerase chain reaction (RT-PCR) technology, the current study examined whether B-CLL cells express immunoglobulin (Ig) receptors that share features of CAs and whether signs of intrachonal diversification and ongoing antigenic selection can be found. Notably, a light-chain–restricted, antigen-independent intrachonal diversification was found in IgM*CD5* B-CLL cells expressing mutated \( V_{H}5-34/VA27 \) gene rearrangements.

**Study design**

**Patient**

A 56-year-old man sought treatment for a 1-year history of cold-dependent acrocyanosis and acral numbness. Primary CAD was diagnosed by the detection of high-titer (1:4000 at 4°C) monoclonal IgM/\( Ig_{\mu} \) CA and the exclusion of CA syndrome secondary to another disease, especially lymphoma. CA specificity was determined as anti-I by reactivity against panels of group O erythrocytes. Results of direct antiglobulin testing using anticomplement sera were positive, and mild hemolytic anemia was observed. The patient responded to the prevention of cold exposition, repeated plasma exchange, and administration of low-dose prednisolone.

During a 7-year follow-up, the paraproteinemia level increased slowly, whereas IgG serum levels (10 to 5.5 g/L) decreased. Increasing CA titers (up to 1:512 000) but moderate autoimmune hemolytic anemia (hemoglobin count, 10.4 g/dL) accompanied worsening of the acral symptoms. Platelet count was normal. Increased and sustained absolute levels of small, mature-appearing lymphocytes (6.5 × 10^9/L–7.2 × 10^9/L) with coarsely clumped chromatin were observed in the peripheral blood. In the bone marrow, erythroid hyperplasia and nodular infiltrates of CD20+/CD23+/CD5-/IgM+ lymphoid cells (accounting for approximately 40% of all nuclear cells) were detected. The diagnosis of B-CLL was confirmed by flow cytometry and by analysis of IgV gene rearrangements. Approval was obtained from the institutional review board of the Charité University Hospital for these studies. Informed consent was provided according to the Declaration of Helsinki.

**Cytometric analysis**

Peripheral blood mononuclear cells (PBMCs) were prepared as described from heparinized venous blood samples kept at 37°C. CD19+ B cells were enriched by negative immunomagnetic separation (Miltenyi Biotec, Bergisch- Gladbach, Germany). Incubation with antibodies, propidium iodide staining, and flow cytometric analysis were performed as reported previously.

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Results and discussion

The demonstration of an expanded monotypic B-cell population is central to the diagnosis of B-CLL. In this study, the diagnosis of B-CLL was confirmed by the detection of a markedly expanded CD19+/CD20+/CD5+/CD27+/sIgM+ expression in a peripheral blood population (comprising approximately 98% of all CD19+ cells), including a striking κ light-chain restriction (Figure 1).7-9 No sIgG, sIgD, or sIgA was detected.

By single-cell RT-PCR, these leukemic B cells were found to express mutated IgVH/Vκ gene pairings, indicating their memory cell-type origin.17,18 Twenty-eight clonally related cells were analyzed, each encompassing a mutated VH+34 gene segment (mutational frequency, 7.7%) rearranged to D3-22 and Jκ3 segments, with a unique 42-base pair (bp) complementarity determining region (CDR3). Invariably, all of these VH rearrangements shared the same mutations. The ratios of replacement (R) and silent (S) mutations determined for the FRs and CDRs were 4:7 (0.6) and 4:1 (4.0), respectively. A significant scarcity of FR R mutations, as observed in the patient’s leukemic heavy-chain transcripts, is regarded to reflect negative selection of R mutations in these regions to maintain the functional stability of the expressed immunoglobulin molecule.16

Coexpressed light-chain transcripts were identified in 21 of the 28 analyzed leukemic B cells. All expressed mutated VκA27-Jκ2 rearrangements, with the same 27-bp CDR3 lacking any indication of TdT activity. Twelve light-chain rearrangements displayed an identical mutational pattern (mutational frequency, 4.8%). The R/S ratios determined for FRs and CDRs were 5:1 (5.0) and 3:2 (1.5), respectively. Most notably, 9 rearrangements displayed additional bp exchanges in their FRs or CDRs (Figure 2a) that were confirmed by several PCR replications from the original wells. These nucleotide exchanges appeared to reflect ongoing mutations because their frequency (13/5271 bp; 2.5 × 10−3/bp) was significantly higher than the estimated PCR error rate (1 × 10−5/bp; P < .001, χ2 analysis), and no variations were found in 7420 bp on the heavy-chain level. The intraclonal relationship of cells expressing diverse VκA27 transcripts is given in a genealogical scheme19 (Figure 2B). To further delineate the origin of these clonally related cells, the presence of the protooncogene bcl-2 mRNA was analyzed by specific nested PCR and DNA sequencing. Notably, bcl-2 transcripts were detected in invariant and variant clonally related single cells in the stage of B-CLL, further indicating their relationship to the same population. By contrast, clonally related single-sorted B cells (4 of 96; 4.2% of sorted cells) obtained before the diagnosis of B-CLL did not express bcl-2 transcripts. Moreover, enriched CD19+ B cells obtained at the time of B-CLL were positive for bcl-2 and bcl-2/sIgH fusion transcripts, whereas those collected before the time of B-CLL were negative for both products, suggesting that this transformation step occurred between these time points.20

To our knowledge, this is the first report of intraclonal variable light-chain diversification in IgM+CD5− B-CLL.9,22 However, given that the common and diverse mutations were randomly scattered throughout the VH segments lacking a preferential accumulation of R mutations in the CDRs, the ongoing mutational process seemed to be antigen independent. In this regard, our findings are in line with those of a recent study suggesting that antigen may no...
longer be able to play a significant role in the clonal expansion of CD5+/IgM− B-CLL cells. 22 One explanation for the variable light-chain–restricted intraclonal heterogeneity in our study might be an ongoing antigen-independent but differential mutational process affecting only the light-chain locus—for example, because of differences in accessibility or genetic abnormalities. 23 In this context, preliminary data show that approximately 10% of patients with B-CLL have mutations in their rearranged variable light-chain genes only.18 Whether these findings belong to a distinct subset of patients, however, remains uncertain.

Remarkably, clinical findings, shared immunoglobulin isotypes with the paraprotein, and VH4-34/CDR3/III expression 24-26 suggest that the patient’s leukemic cells had developed from his CA-expressing population. This assumption is supported by the detection of the same monoclonal and unique VH4-34/CDR3/III sequence in the patient’s B cells obtained before diagnosis of B-CLL lacking bcl-2/IgH fusion transcripts and in B cells obtained at the stage of B-CLL with bcl-2/IgH translocation. Notably, VH4-34 expression is crucial in the specific recognition of the anti-I/i system, 24-26 whereas the CDR3 and light chain are thought to modify the fine specificity and affinity of binding. 26 Moreover, expansions of CD5+/IgM+ B cells in CAD 6,27 and, conversely, B-CLL cells producing CA 2 have been reported. Although antibodies using VH4-34 do not necessarily have anti-I/i specificity 25 and although VH4-34 usage in IgM− B-CLL cells is frequent, 17,18 selection by the I-antigen might have contributed to the distinct mutational pattern of the patient’s expanded CD5−IgM+ B-cell population. In conclusion, single-cell RT-PCR provides a powerful tool for analyzing IgV gene expression in cellular (sub)populations of heterogeneous lymphoproliferative diseases, such as B-CLL, to enable further insight into the association with known autoimmune phenomena.

References


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