Clonal Heterogeneity Assessed by Flow Cytometry in B-Cell Lymphomas Arising From Germinal Centers

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Key Words: Flow cytometry; Follicular; Lymphoma; Transformation; Burkitt lymphoma; Diffuse large cell lymphoma

Abstract

Patients with mature follicular B-cell lymphomas develop aggressive non-Hodgkin lymphomas (NHLs) during disease progression. It is controversial whether most diffuse large B-cell lymphomas (DLBCLs) and Burkitt lymphomas (BLs) emerge as de novo lymphomas or from an original follicular lymphoma. To distinguish clonally related populations in aggressive NHL, we studied the immunophenotypic features of 18 consecutive samples from 16 patients. Three flow cytometric patterns were distinguished: (1) a homogeneous neoplastic population of large B cells with phenotypic features of follicular center cells; (2) 2 atypical populations of B cells, small monoclonal B cells, and large B cells with loss of some surface antigens; and (3) 2 clonal populations of small and large B cells sharing the same light-chain isotype. The 3 flow cytometric patterns were observed, respectively, in de novo DLBCL and BL, transformation into BL, and transformation into DLBCL. Flow cytometric data can provide valuable information about the natural history of NHL.

Non-Hodgkin lymphomas (NHLs) arising from germinal centers may display different morphologic features. Three categories are distinguished according to the World Health Organization (WHO) classification: follicular lymphomas (FLs) graded by the number of large cells and the presence of diffuse areas composed of large cells, diffuse large B-cell lymphomas (DLBCLs), and Burkitt lymphomas (BLs). These entities share morphologic, phenotypic, and molecular features. DLBCLs and BLs can emerge de novo or as a result of transformation from FLs. These lymphomas show phenotypic features of follicular B cells, ie, positivity against CD19, CD22, and CD10, but there is no specific immunophenotypic profile associated with each category. Heterogeneous molecular lesions have been identified, ie, rearrangements of the bcl-2 proto-oncogene due to the t(14,18) (q32;q21) chromosomal translocation in FLs, rearrangements of the bcl-6 proto-oncogene in de novo DLBCLs, and c-myv rearrangements in BLs. However, these are not specific to one entity and have been observed during the progression and transformation of FLs into more advanced lymphomas. Overlapping biologic features exist among these lymphomas.

According to their natural history, aggressive NHLs can be separated into de novo and transformed NHLs. Commonly, the existence of a lymphoma history and conventional molecular methods showing the same V(D)J junction rearrangement in small and large malignant B lymphocytes has been used to demonstrate the transformed origin of an aggressive NHL. However, the introduction of multiparametric techniques in hematology, such as flow cytometry, enables a rapid detection of different subsets of clonally related cells from 1 sample in a single procedure. We studied samples from 16 patients with aggressive NHLs with...
or without a history of FL. The aims of this work were to define the usefulness of flow cytometry as a routine laboratory method to identify different B-cell clones and to deduce the natural history of NHLs with a common origin in germinal centers.

Materials and Methods

Patient and Sample Selection

Between January 1997 and December 2000, 18 samples from 16 consecutive adult patients were studied. Samples were chosen if they had immunophenotypic features of follicular center origin and if they represented DLBCL and BL according to the WHO classification. Monoclonal was defined by flow cytometry when there was evidence of surface immunoglobulin (sIg) light-chain restriction, although a homogeneous lack of expression of sIg associated with an abnormal immunophenotype (ie, not detected in normal B cells) was regarded as a signal for the malignant nature of the B cells. Samples were peripheral blood (n = 3), bone marrow (n = 10), and lymph node cell suspensions (n = 5). In all cases, the fraction of malignant cells was 50% or more. One case (case 7) showed a posttransplant lymphoproliferative disorder with a BL diagnosis. The samples were studied by cytologic and histologic analysis and by immunophenotyping. All samples were examined for the presence of bcl-2 rearrangements using a long-distance polymerase chain reaction (PCR) approach, and cases with a diagnosis of BL were analyzed for the presence of c-myc gene rearrangements by Southern blot analysis. A conventional cytogenetic study was performed in 7 cases (2, 3, 5, 8, 10, 13, and 14). All samples were obtained at diagnosis, and dates, type of sample, and percentage of infiltration were recorded.

Morphologic Assessment

Morphologic features of these lymphomas were assessed on lymph nodes, bone marrow sections, and peripheral blood samples using standard techniques. Slides were stained with H&E for light microscopy. On the basis of the WHO classification, samples were classified as BL (n = 11) or DLBCL (n = 5). BL showed the characteristic cytologic (vacuolated and basophilic cells) and histologic picture (a “starry sky” pattern) associated with a very high growth fraction (>96% Ki-67+ cells).

In lymph node biopsy specimens, an immunochemical analysis of CD20, CD10, CD79a, Ki-67, CD5, and CD3 monoclonal antibodies (purchased from DAKO, Glostrup, Denmark) and bcl-2 and bcl-6 protein expression was performed. The reaction for bcl-2 was classified as positive when more than 10% of the tumor cells showed cytoplasmic staining.

Flow Cytometric Analysis

Peripheral blood, bone marrow, and freshly disaggregated lymph node suspensions were analyzed without additional preparation. The number of cells was quantified by microscopy and adjusted to 2 x 10⁶ in each tube. For sample preparation, a stain, lyse, and wash procedure was used; erythrocytes were lysed by means of FACS Lysing solution (Becton Dickinson [BD], San Jose, CA). Clonality study of B lymphocytes was undertaken using a triple reagent consisting of a combination of kappa–fluorescein isothiocyanate (FITC), lambda–phycoerythrin (PE), and CD19-PE/Cy5 in a single tube (kappa/lambda, Simultest, BD; CD19, Caltag, San Francisco, CA). Each clonal sample was studied using a panel of triple monoclonal antibodies (FITC, PE, PE/Cy5): FMC7-FITC (FMC7, Harlan Sera-Lab, Belton, England)/CD5-PE (L17F12, BD)/CD19-PE/Cy5 (SJ25C1, Caltag); CD22-FITC (S-HCL-1, DAKO)/CD23-PE (EBVCS-5, BD)/CD19-PE/Cy5 (Caltag); CD103-FITC (B-Ly7, Immunoquality, Groningen, the Netherlands)/CD25-PE (ZA3, BD)/CD19-PE/Cy5 (Caltag); CD10-FITC (W8E7, BD)/CD11c-PE (D 12, BD)/CD19-PE/Cy5 (Caltag); and CD79b-FITC (SN8, DAKO)/CD20-PE (L 27, BD)/CD10-PE/Cy5 (HI10a, Pharmingen, San Diego, CA).

Measurements were performed on a FACScan flow cytometer using the LYSIS-II software for data acquisition and the PAINT-A-GATE PRO software for data analysis (BD). The analysis was performed on gated lymphoid cells, and although 15,000 events per tube were measured in all samples, CD19- cells were selectively introduced for clonality analysis. The positivity threshold was 20% for all markers.

Molecular Studies

DNA extraction was performed by digestion with Proteinase K, extraction by the “salting out” method, and precipitation with ethanol. A long-distance PCR approach using the protocol published by Akasaka et al was used to study bcl-2 rearrangements. Briefly, we used 100 ng of genomic DNA, a 2.5-mmol/L concentration of each deoxynucleoside triphosphate, 20 pmol of each primer, and 2.5 U Tag DNA polymerase (Takara LA Taq polymerase, Takara Shuzo, Kyoto, Japan), in a final volume of 50 µL. Long-distance PCR conditions were as follows: 1 cycle of denaturation (94°C for 1 minute); 30 cycles of denaturation (98°C for 20 seconds), and annealing (68°C for 20 minutes) followed by a cycle of extension (72°C for 16 minutes). The sequence of the primers used to detect the immunoglobulin/bcl-2 fusion genes has been reported previously.

Southern Blot Analysis

Five micrograms of DNA were digested using EcoRI and HindIII (New England Biolabs, Beverly, MA) restriction enzymes. DNA was separated on a 0.7% agarose gel and...
transferred to nylon membranes (Amersham, Buckinghamshire, England), which were hybridized with the MC413RC probe labeled with phosphorus 32–deoxycytidine triphosphate by the random primer extension method. Filters were washed in 0.2× sodium chloride–sodium citrate/0.5% sodium dodecyl sulfate for 2 hours at 65°C and then autoradiographed using intensifying screens.22

Cytogenetic Analysis

Cytogenetic studies were undertaken on fresh lymphoma cells after short-term culture without stimulation. Chromosomes were banded using G banding, and chromosomal abnormalities were described according to the International System for Human Cytogenetic Nomenclature.23

Results

Patient Characteristics

We studied 18 samples from 16 patients. Two samples were analyzed in 2 cases (11 and 12) Table 1. All patients were adults, 7 men and 9 women. The disease stage at diagnosis was III or IV in all cases. All patients with DLBCL were categorized as having a high-risk international prognostic index (score of 4-5). Five patients had extranodal involvement at diagnosis. The extranodal sites affected were the leg, pelvis, retroperitoneal mass, intestine, and heart. Even in these cases, there was a concomitant lymph node enlargement that was biopsied to establish the diagnosis. Test results for HIV were positive in 4 cases (Table 1). Only 2 patients remained alive 5 months after the diagnosis. One of these was diagnosed with a posttransplant BL.

Flow Cytometric Results

In all samples, neoplastic B cells showed phenotypic features of follicular cells.18 They showed positivity against CD10-PE/Cy5 and/or CD10-FITC, CD20, CD22, CD19, HLA-DR, and cCD79a, with negativity against CD5, CD34, and terminal deoxynucleotidyl transferase. Interestingly, all samples were negative for CD23, an antigen commonly expressed by mature B cells.18 Five lymphoma samples showed no sIg expression. The immunophenotypic profile of cases in which a normal population of B cells was detected revealed positivity for FMC7, CD19, CD20, CD22, CD23, and HLA-DR and negativity for CD5, CD10-PE/Cy5, and CD10-FITC with a polyclonal expression pattern of sIg.

We found 3 immunophenotypic patterns according to the antigenic profile of malignant B cells Figure 1: (1) Pattern 1 was a homogeneous monoclonal population of large B cells

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BL, Burkitt lymphoma; BM, bone marrow; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; FSC, forward scatter; G, germinal; LN, lymph node; ND, not done; PB, peripheral blood; R, rearranged; sIg, surface immunoglobulin; +, weak; ++, moderate; ++++, strong; –, negative.

* See the “Results” section for an explanation of the immunophenotypic patterns.
(high forward scatter [FSC]–side scatter [SSC] distribution) (12 samples from 11 patients; cases 4, 6-11, and 13-16). This pattern was considered as representative of de novo aggressive NHL, BL, or DLBCL. Three samples from 3 patients with BL did not express sIg, whereas samples from all patients with DLBCL showed sIg expression (Figure 1A). (2) Pattern 2 included 2 atypical subpopulations of B cells constituted by small and large B cells (cases 1-3). The first subpopulation corresponded to a small clonal B-cell population, whereas the other subpopulation represented larger atypical cells; these larger cells had some different immunophenotypic features with respect to the small B-cell population (sIg and CD23 loss and CD10-FITC gain) (Figure 1B). (3) Pattern 3 included 2 monoclonal subpopulations of B cells showing the same light-chain isotype (3 samples from 2 patients; cases 5 and 12). These 2 subpopulations could be distinguished because one of them was reactive against CD10-PE/Cy5 but not against CD10-FITC, and it had low FSC/SSC distribution, whereas the other subpopulation showed CD10-FITC and CD10-PE/Cy5 expression with high FSC/SSC distribution and loss of CD23 reactivity (Figure 1C). The former population was compatible with a low-grade lymphoma, and the second corresponded to transformation to DLBCL.

These atypical patterns should be distinguished from the simultaneous detection of mature normal B cells and normal B-cell precursors in bone marrow samples. Normal precursors did not show atypical expression of B-cell differentiation antigens or expression of sIg. Mature normal B cells showed negativity against CD10-FITC (W8E7) and a polyclonal sIg pattern (Figure 2).

**Correlation Among Clinical, Morphologic, Phenotypic, and Molecular Findings**

Samples classified within immunophenotypic pattern 1 could be separated into 2 categories according to morphologic features and medical history: (1) de novo DLBCL (n = 3;
cases 6, 15, and 16) and (2) de novo BL (n = 8; cases 4, 7-11, 13, and 14). De novo DLBCLs were distinguished from de novo BLs by flow cytometry because large B cells of the DLBCL cases showed positivity against CD10-PE/Cy5 with negativity against CD10-FITC, and they showed homogeneous expression of sIg. In cases of de novo BL, samples were positive for both CD10-PE/Cy5 and CD10-FITC, with some cases showing lack of expression of sIg. Analysis of bcl-2 rearrangements in this group demonstrated the t(14;18) chromosomal translocation in 2 of 11 cases, one being diagnosed as de novo BL (case 8) and the other as de novo DLBCL (case 15). Involvement of the 8q24 band was found in 3 of 8 cases within this group (cases 4, 8, and 13), but we found no association with a specific antigenic profile. Simultaneous detection of the t(14;18) and t(8;14) chromosomal translocations was found in 1 case (case 8).

Of 3 cases classified in the flow cytometric pattern 2 (transformation to BL), 2 characteristically showed loss of some surface antigens at the time of transformation (CD20, sIg, or CD23). One case (case 2) showed a t(8;14) chromosomal translocation, and another case (case 3) showed complex cytogenetic abnormalities associated with the t(14;18)(q21;q32). The complex karyotype suggests the accumulation of several molecular lesions in different steps during the progression and transformation from mature B cells to a blastic phase.

The 2 cases with flow cytometric pattern 3 (n = 3 samples) showed bcl-2 rearrangements resulting from the t(14;18)(q21;q32) chromosomal translocation, and all patient samples showed sIg expression in small and large B cells, as evidenced by flow cytometry.

**Discussion**

The immunophenotypic changes associated with FL transformation have not been characterized clearly. To determine the usefulness of flow cytometry in distinguishing de novo from transformed aggressive NHL, we analyzed the immunophenotypic features of different B-cell subpopulations in DLBCL and BL samples. We found the following immunophenotypic traits that could be associated with the different biologic behavior of these lymphomas: (1) CD10-FITC (W8E7) was expressed commonly by aggressive NHL (de novo and transformed). (2) In the cases in the present series with 2 clonal B-cell populations (small and large cells), the small cells were CD23+, and the large cells were CD23−, possibly suggesting that CD23 was lost during the transformation into DLBCL or BL. CD23 is a molecule that recognizes the lymphocyte low-affinity receptor for IgE (Fc epsilon RI), an antigen involved in B-cell differentiation. (3) The detection of 2 monoclonal populations of small and large CD19+ cells in the same sample with the same isotype of sIg suggests a common single-cell origin (Figure 1). (4) Detection in the same sample of small B lymphocytes with monoclonal expression of sIg and large B cells with lack of sIg expression could be a sign of transformation into an aggressive lymphoma, especially when it is associated with other antigenic losses (ie, CD23).

The histologic features of cases classified as transformed DLBCL or BL were reviewed in comparison with earlier tumor samples from the same patients, and the diagnosis of transformed NHL was confirmed.

Comparison of de novo and transformed aggressive lymphomas also revealed common immunophenotypic features, reflecting the same origin in germinal centers. Both entities showed an immunophenotypic profile typical of mature B cells of follicular center origin,25,26 i.e., positivity against CD19, CD20, CD10-PE/Cy5, CD22, and CD23, whereas they showed variable positivity against the FMC7 and CD79b monoclonal antibodies. Flow cytometry is, thus, a very useful complementary tool to study biologically aggressive variants of FL.

Flow cytometry has been used to analyze different populations of B cells in cases of Richter syndrome.26,27 These studies reported a common immunophenotype in mature and
transformed B cells of chronic lymphocytic leukemia, suggesting a common origin in a single progenitor cell. We report our results using triple immunostaining and confirm the usefulness of flow cytometry as a diagnostic method to study subclones in FL and transformed aggressive NHL.

Five cases classified as de novo BL did not show slg expression (Table 1). The lack of expression of slg is an uncommon but a previously reported finding in NHLs. It is believed that the lack of slg expression reflects a posttranscriptional defect, given that rearrangements of the heavy-chain immunoglobulin gene have been evidenced in some of these cases. This finding is common in cases of mediastinal large B-cell lymphoma with sclerosis, nonfollicular center cell lymphomas, and chronic lymphocytic leukemia. BLs also may lack the expression of slg measured by flow cytometry as shown in this study. In these cases, slg loss also could be a sign of the apoptotic process.

Detection of various populations of B cells also occurs in a substantial proportion of bone marrow samples that show normal progenitors of B lymphocytes and in cases of peripheral blood samples of leukemic NHL. In bone marrow samples of BL or DLBCL, the detection of CD34+/terminal deoxynucleotidyl transferase+/CD23−/CD20−/slg− cells with lack of slg expression identifies normal B-cell progenitors. The normal B-cell compartment in peripheral blood samples in cases of leukemic NHL is characterized by a clonality assay showing a polyclonal slg pattern and normal positivity for CD20 and CD23. The present study revealed the coexistence of normal and neoplastic B lymphocytes in 4 cases (2 bone marrow and 2 peripheral blood samples). The identification of these B-cell populations also was used as an indirect marker of the level of peripheral blood contamination and the residual hematopoietic normal tissue in bone marrow samples.

The t(8;14)(q24;q32) and t(14;18)(q32;q21) chromosomal translocations are frequent in BL and FL, respectively, but they also have been observed in a proportion of cases of DLBCL. It is noteworthy that all lymphoma cases with the t(14;18)(q32;q21) showed slg expression. In patients with de novo BL, 50% of cases (4/8) showed HIV positivity, and 2 of these 4 showed c-myc rearrangements when a third exon probe was used. This underscores the importance of testing for HIV infection in all cases of BL. The coexistence of the two t(8;14) and t(14;18) chromosomal translocations was observed in only 1 patient (case 8). Although the detection of the t(14;18) chromosomal translocation could suggest transformation from a previous FL, this patient had a diagnosis of de novo BL, and he had no medical history of lymphoma. Recently, the detection of the t(14;18) chromosomal translocation has been reported in de novo hematologic malignant neoplasms.

These findings suggest that although de novo aggressive NHLs are phenotypically similar to transformed BLs or DLBCLs, different immunophenotypic patterns may be associated with these categories. A clear definition of the various immunophenotypic patterns that occur in aggressive NHL is a key to understanding the natural history and biologic behavior of these diseases. Flow cytometry could be a useful complementary tool for distinguishing the nature of NHLs and for designing better therapeutic strategies.

References


