Flow Cytometric Sorting of Paraffin-Embedded Tumor Tissues Considerably Improves Molecular Genetic Analysis

Ekaterina S. Jordanova, MSc, Willem E. Corver, PhD, Marcel J. Vonk, Math P.G. Leers, PhD, Sietske A. Riemersma, MD, Ed Schuuring, PhD, and Philip M. Kluin, MD, PhD

Key Words: Paraffin-embedded tissue; Flow cytometric sorting; B-cell lymphoma; HLA; Loss of heterozygosity; LOH; Flow cytometry; Fluorescent in situ hybridization; FISH

DOI: 10.1309/HPR11R7LQ9NNCCG8

Abstract

The characterization of genetic aberrations in paraffin-embedded tumor material is impaired by contaminating normal cells. In the present study on the genetic causes of loss of HLA expression in diffuse large B-cell lymphoma (DLBCL), we compared the efficacy of microdissection with flow cytometric sorting of tumor cells. Single-cell suspensions from paraffin-embedded material of 5 DLBCL cases were stained for CD79a and DNA content (propidium iodide). Fluorescent in situ hybridization (FISH) using HLA class II and chromosome 6 centromeric probes and loss of heterozygosity (LOH) analysis with 5 HLA-specific microsatellite markers were performed on microdissected and flow cytometry–sorted fractions. FISH confirmed considerable enrichment of the samples after flow cytometric sorting and disclosed tumor heterogeneity in 4 cases. Moreover, lymphomas with a so-called zebra LOH pattern in the microdissected material showed unambiguous LOH after flow cytometric sorting, revealing in 1 case a biologically relevant hemizygous deletion in the HLA region.

Hemizygous and homozygous deletions of HLA class I and class II genes are a common event in diffuse large B-cell lymphoma (DLBCL) of the central nervous system (CNS) and the testis. Subsequent loss of HLA expression is thought to be a major tumor escape mechanism facing antitumor immunity, as these molecules present tumor-specific peptides to cytotoxic T lymphocytes. However, a difficulty encountered during these studies was the presence of high amounts of infiltrating normal cells, impairing the identification of the precise genetic aberrations that led to loss of expression. In particular, loss of heterozygosity (LOH) studies on unpurified or grossly microdissected tumor fractions often resulted in alternate patterns of LOH, retention of heterozygosity, and/or LOH with low imbalance factor. This is referred to as a “zebra pattern,” which might be because the individual markers are affected differently by the admixture of normal cells.

In the present study, we adapted and modified a method previously described for isolation and flow cytometric analysis of single cells from archival paraffin-embedded tumor material to establish optimal conditions for studying DLBCL tissue. To this end, the protocol was adapted for the CD79a antigen, which is expressed on the cell surface and within the cytoplasm of almost all normal and neoplastic B cells. Five cases of DLBCL with known aberrations of the HLA region on 6p21 but LOH patterns that were difficult to interpret were selected. Interphase fluorescent in situ hybridization (FISH) using a P1 artificial chromosome (PAC) probe located in the HLA region and a chromosome 6–specific centromeric probe enabled us to validate the enrichment of tumor cells with 6p21 deletions. Our data show that flow cytometric sorting is
very helpful for genetic analysis of paraffin-embedded lymphoma tissue and is superior to gross microdissection of tumor cells.

**Materials and Methods**

**Tissue Samples**

The 5 cases used in this study were selected from a previously described series. The B-cell origin of the 5 DLBCL cases was confirmed by immunohistochemical staining for CD19, CD20, CD22, or CD79a. One lymphoma was of primary cerebral origin, and 4 were of primary testicular origin. Formalin-fixed, paraffin-embedded tissue blocks from these cases were obtained from the tissue bank of the Pathology Department, Leiden University Medical Center, Leiden, the Netherlands, and from the Josephine Nefkens Institute, Rotterdam, the Netherlands.

**Preparation of Cell Suspensions for Flow Cytometric Analysis**

The method for DNA flow cytometric analysis of single-cell suspensions from paraffin-embedded material, as described by Leers et al., was optimized for DLBCL. Two sections of 50 µm were cut from 5 formalin-fixed, paraffin-embedded DLBCL cases and placed in 10-mL glass tubes. The sections were deparaffinized 3 times for 5 minutes in 3 mL of xylene and rehydrated in a descending ethanol range. After a wash in 3 mL of phosphate-buffered saline (PBS), the sections were immersed in 3 mL of cold citrate solution (10-mmol/L, pH 6.0) and incubated for 80 minutes at 80°C in a water bath. The sections were permitted to cool to room temperature, centrifuged (5 minutes, 500g), and digested for 10 minutes at 37°C in a water bath in 3 mL of a 0.05-mol/L concentration of tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer complemented with a 10-mmol/L concentration of calcium chloride and a 0.1% trypsin solution (Sigma Diagnostics, St Louis, MO). We then added 3 mL of PBA (PBS containing 1% bovine serum albumin; Sigma) to block the digestion, which was followed by a mechanical treatment, mincing with a pipette tip. Next, the samples were filtered through a 50-µm mesh nylon filter and washed 3 times in 3 mL of PBA (5 minutes, 500g).

The cell pellet was resuspended in PBA, and the single-cell suspension was divided into aliquots of 1 × 10⁶ cells. To each sample, 100 µL of mouse-antihuman CD79a antibody (450 µg/mL; DAKO A/S, Glostrup, Denmark) diluted 1:40 in PBA was added. After overnight incubation at room temperature, the samples were washed twice in PBA. Next, 100 µL of the secondary antibody goat F (ab2)-antimouse-IgG1 RPE (R-phycocerythrin; 250 µg/mL; Southern Biotechnology Associates, Birmingham, AL) diluted 1:100 in PBA was added to the pellet. Incubation of 30 minutes in the dark was followed by washing 3 times with 3 mL of PBA. DNA staining solution was prepared in PBA, containing 0.1% ribonuclease A (RNase; Sigma) and a 1-µmol/L concentration of propidium iodide (PI; Calbiochem, San Diego, CA). Owing to a significant spectral overlap with RPE, we used a relatively low concentration of PI. The applied antigen retrieval method was not hampered by the lower PI concentrations. Cells were incubated with 0.5 mL of DNA staining solution for 30 minutes at 37°C to activate the RNase. The samples were kept at 4°C for at least 2.5 hours in the dark before flow cytometric analysis and sorting. Negative control samples omitting the primary antibody were treated the same way.

**Flow Cytometry and Sorting**

For each measurement, data from 10,000 to 20,000 single-cell events were collected using a FACS caliber flow cytometer (BD Biosciences, San Jose, CA) equipped with a 15-mW Argon-ion laser. RPE (FL2, BP 585/42 nm, orange fluorescence channel) fluorescence measurements were collected in the logarithmic mode. PI (FL3, LP 670 nm, red fluorescence channel) fluorescence was collected in the linear mode. A threshold was set on the forward scatter parameter during acquisition. We applied an FL3-area vs FL3-width pulse processing gating strategy during the analysis to discriminate between single cells and debris (nuclear fragments) and cell aggregates, which strongly enhances the enrichment for single intact nuclei. Data were analyzed using the WinList 4.0 and ModFit LT 3.0 software packages (Verity Software House, Topsham, ME).

Flow cytometric sorting was performed using a FACStar Plus flow cytometer (BD Biosciences). A life gate was set on the FL3-area vs FL3-width pulse processor. Cells were sorted based on CD79a expression (RPE) and DNA content (PI). Both positive and negative cell fractions were collected for DNA extraction as well as directly sorted onto glass slides for FISH processing.

**DNA Extraction**

For LOH analysis, tumor and nontumor cells were separated by microdissection of 10-µm paraffin-embedded tissue sections stained with H&E. Before the dehydration steps, the procedure was interrupted for microdissection. Tumor areas with an estimated content of 70% or more tumor cells were microdissected using a needle under direct microscopic visualization. Normal cells were obtained by using the same procedure. DNA was extracted.
from microdissected cells of the 5 DLBCL cases by Proteinase K (50 µg/mL) digestion at 56°C, phenol-chloroform extraction, and ethanol precipitation as previously described. The obtained DNA pellet was dissolved in Tris-EDTA (10-mmol/L Tris, 0.1-mmol/L EDTA, pH 7.6). We used 1 µL as a template for the polymerase chain reactions (PCRs).

DNA from the flow cytometry–sorted tumor and normal cells of the 5 DLBCL cases was extracted by the method described by Abeln et al. In short, flow cytometry–sorted tumor cells and normal cells were resuspended in isolation buffer (0.3 mg/mL of Proteinase K in 10-mmol/L Tris hydrochloride, pH 8.3; a 1-mmol/L concentration of EDTA; 0.5% solution of Tween 20) at a concentration of 1,000 cells per microliter. After incubation overnight at 56°C in a water bath and a 5-minute inactivation at 100°C in a heat block, 1,000 cells were used as a template for the PCRs.

LOH Analysis

DNA from microdissected and flow cytometry–sorted tumor and normal material was analyzed for LOH by PCR amplification. Each case was studied by using microsatellite markers located in the HLA region on chromosome 6p21.3 that previously had shown ambiguous results for the microdissected material (C125, TY2A, TNF-α [tumor necrosis factor α], D6S273, C47). The primer sequences for the microsatellite markers and reaction conditions have been described. Standard PCR amplifications were carried out, and 1 µCi [α-phosphorus 32]-αCTP (American, Buckinghamshire, England) was incorporated. The radiolabeled PCR products were analyzed on a 6% polyacrylamide gel, and dried gels were autoradiographed. The Molecular Dynamics Phosphor Imager 445SI (Molecular Dynamics, Sunnyvale, CA) was used for quantification of the autoradiograms. An imbalance factor of 1.7 or more was considered LOH. An imbalance factor between 1.4 and 1.7 was regarded as “borderline” LOH and was depicted separately.

Interphase FISH Analysis

Interphase FISH analysis was performed on nuclei isolated from formalin-fixed, paraffin-embedded tissue of all 5 cases using an HLA region class II–specific PAC clone (172K2) and a probe for centromere 6 as previously described. The α-satellite centromeric 6-probe (D6Z1), labeled with biotin-16-deoxyuridine triphosphate (dUTP; Roche Diagnostics, Mannheim, Germany), was provided by J. Wiegent (Department of Molecular Cellbiology, Leiden University Medical Center). The PAC probe was labeled with digoxigenin-12-dUTP (Roche Diagnostics) by standard nick translation. Hybridization and immunodetection were performed as previously described.

For interphase FISH on flow cytometry–sorted cell fractions, the protocol was modified. Approximately 400 cells were flow cytometry–sorted directly onto glass slides that were cleaned by rinsing in 96% ethanol. The slides were dried overnight at room temperature to ensure adhesion of the cells. If needed, the slides were incubated in a 0.1-mol/L solution of Na2B4O7 to permit swelling of the nuclei. Afterwards, the slides were rinsed in PBS and sterile water. The cells were fixed on the slides by incubating them in 4% formaldehyde in PBS for 5 minutes. Finally, the slides were washed 3 times for 5 minutes each in PBS, dehydrated, and air dried. Hybridization and immunodetection were performed in the same way as for nuclei isolated from paraffin-embedded material.

Results

Flow Cytometry and Sorting

Microscopic examination of cytocentrifuged cells isolated from paraffin-embedded material for flow cytometry showed that the majority of nuclei had retained some cytoplasmic remnants, which would be sufficient for binding of the CD79a antibody, especially since the majority of the CD79a protein is retained in the endoplasmic reticulum. The combined enzymatic and mechanical treatment resulted in a yield ranging from 5 to 20 × 10⁶ cells. In all cases, the tumor fraction expressing CD79a could be distinguished readily. The percentage of CD79a+ cells ranged from 5% (case T26) to 85% (case C11), which is indicative of the presence of variable numbers of non–B cells in the sample. In Figure 1 the flow cytometry results for 2 representative cases are depicted.

Interphase FISH Results

By implementation of interphase FISH, we found that flow cytometric sorting of CD79a+ cells leads to substantial enrichment of the tumor samples. In Figure 2, we found that flow cytometric sorting of CD79a+ cells leads to substantial enrichment of the tumor samples. In Figure 2, we detected 15% of the cells carrying a hemizygous deletion. After flow cytometric sorting, this percentage was 29%. The most dramatic increase was observed for the fraction of tumor cells with 3 centromere 6 signals and 2 signals for the PAC probe, which increased from 7% to 60%. Similarly, in cases T16 and T22, the percentages of cells with deletions increased more than 2-fold after flow cytometric sorting. In Figure 2, we found that flow cytometric sorting of CD79a+ cells leads to substantial enrichment of the tumor samples.
cells lacking deletions in the HLA region. Alternatively, although not seen in (immuno)histology and highly unlikely in these primary extranodal lymphomas without preexisting inflammation, we cannot exclude that this tumor contained many CD79a-expressing reactive B cells or plasma cells.

In case C11 with 85% CD79a+ cells, we detected 14% normal cells before and 5% after flow cytometric sorting, indicating that this tumor was relatively pure. Before and after sorting, respectively, 40% and 50% of the cells showed 5 signals for both the centromere 6 and the PAC probes. In this particular case only, DNA aneuploidy also was detected by flow cytometric analysis of DNA content, and it was exclusively present in the CD79a+ fraction (C11, Figure 1B). The normal cell population (CD79a–) did not show any aberrations with FISH (data not shown).

**Allelotyping Analysis of 6p21.3**

The results obtained for LOH analysis on microdissected and flow cytometry–sorted, paraffin-embedded DLBCL material are summarized in Figure 3. Of the 5 cases, 4 showed an LOH zebra pattern before flow cytometric sorting. In cases T16, T22, and T26, enrichment of the tumor cells had a profound effect on the detection of LOH (Figure 3A). In case T16, markers TY2A and C47 showed unclear results before but unambiguous LOH results after flow cytometric sorting. The same was true for marker TNF-α in case T26. In the microdissected material of these 2 tumors, we failed to detect any LOH for the majority of markers; however, after enrichment by flow cytometric sorting, all markers showed LOH.

In case T13, markers C125 and TNF-α showed an allelic imbalance factor between 1.4 and 1.7 before sorting, whereas all other informative markers, including C47 (data for other markers not shown), displayed higher levels of LOH (markers TY2A and D6S273 were homozygous in this case). The results for C125 and TNF-α did not improve in flow cytometry–sorted cells, apparently because of the presence of very strong shadow bands of the upper alleles, which interfered with the detection of a higher LOH factor (Figure 3B). In case C11, no LOH was observed when either microdissected or flow cytometry–sorted material was used.
Discussion

In the present study, we found that flow cytometric sorting of archival paraffin-embedded DLBCL samples using the CD79a antigen is an excellent tool to improve FISH and LOH analysis. We used this technique to study genetic aberrations in the HLA region on chromosome 6p21.3 in 5 DLBCL cases of the testis and the CNS. These lymphomas are characterized by complex hemizygous and homozygous deletions and by mitotic recombination in the HLA region.1 The observed genetic loss frequently accounts for loss of expression of HLA class I and, especially, class II molecules. In B-cell lymphomas, these molecules may present tumor-associated antigens to cytotoxic or helper T lymphocytes, thereby initiating and mediating a potential antitumor immune response. Loss of HLA expression, therefore, may be an important mechanism to escape immune attack. In parallel, in a variety of solid tumors, loss of HLA class I expression is observed frequently (reviewed by Sette et al2).

Purification of tumor cells is especially advantageous for LOH analysis, particularly since admixture with normal cells might yield false-negative results and because the individual DNA microsatellite markers might give variable results. This might give rise to the so-called zebra patterns of LOH when multiple adjacent markers are compared.5 One of the major drawbacks in studying genetic alterations in DLBCL, in particular if found in rare sites such as the testis or CNS, is the prevalent availability of archival paraffin-embedded tumor material. This precludes the preparation of the fresh cell suspensions necessary for conventional multiparameter flow cytometry, purification of tumor cells, and identification of heterogeneous tumor cell subpopulations.

Various techniques ranging from simple microdissection to laser-assisted cell capture have been developed for enrichment or purification of tumor cells from frozen and paraffin-embedded tissue blocks.13,14 However, these methods are not easily applicable to tumors such as lymphomas in which the tumor cells and reactive cells are intermingled and do not form distinct structures. Of note, in the present study, all LOH results for the unsorted tumor and normal cells were obtained after gross microdissection. By using microdissection on H&E-stained sections, we aimed to obtain a tumor sample with at least 70% tumor cells as roughly estimated, but our LOH data suggest that we were not always able to reach that goal. Thus, to improve the LOH data, we applied and modified a recently developed method for isolation of nuclei from paraffin-embedded tumor samples.7 Initially, heat pretreatment of the archival tissue was used for improvement of DNA flow cytometric analysis; however, this method also can be used for flow cytometry of cytoplasmic antigens such as cytokeratin and immunoglobulin light chain proteins and nuclear antigens such as estrogen and progesterone receptors.15-17

Theoretically, DNA content itself could have been used to isolate pure tumor cell populations. Previous reports describe inconsistent percentages of aneuploidy in B-cell lymphoma, varying from a small number to 57% in aggressive lymphomas.18,19 However, in our experience, the incidence of aneuploidy is too low to use this as a useful target, and in the present small series, only 1 of 5 cases showed aneuploidy (Figure 1). Furthermore, although not encountered in the present 5 cases, a relatively high number of cells in the G2 phase of the cell cycle might interfere with the subsequent FISH analysis. We considered CD79a as a candidate target for sorting because this antigen is widely expressed in B cells and because it is expressed not only on the cell membrane but also within the cytoplasm.8 Most likely, cytoplasmic expression is obligatory for this technique because cellular integrity is disrupted during the preparations. Remnants of cytoplasm around the nucleus, therefore, should contain the target for the antibody. An excellent alternative to sort B cells is the nuclear transcription factor Pax-5; however,
it could not be used in this particular study because it is expressed strongly in the testis as well.20,21 For specific lymphoma subtypes, other nuclear antigens such as terminal deoxynucleotidyl transferase or cyclin D1 could be used as well. In the past, terminal deoxynucleotidyl transferase was successfully used as a parameter to sort cells from briefly fixed tissue, and Southern blot analysis on both fractions demonstrated the clonal relationship and different oncogene rearrangements in a composite lymphoma.22

By comparing the interphase FISH results on the nuclei isolated from microdissected and flow cytometry–sorted tumor material, we confirmed a strong enrichment of the tumor cells. Furthermore, intratumor heterogeneity became evident in 4 of 5 lymphomas. As stated previously, LOH results often are inconsistent owing to the presence of normal cells in a tumor sample. The improvement by flow cytometric sorting was demonstrated in case T22, in which all 5 markers showed unambiguous LOH after purification, in contrast with only 1 marker on the grossly microdissected tissue. The biologic significance in this tumor is evident, because after flow cytometric sorting, the LOH proved to extend into the HLA class I region, and the loss of 1 HLA haplotype might be sufficient to contribute to immune escape from HLA class I–restricted CD8+ cytotoxic T cells.

We assert that FISH is an essential supplement to LOH analysis, especially because it is the preferable method to determine whether mitotic recombination or deletion is the mechanism causing LOH and to determine the allelic copy numbers.3,23 Thus, FISH was used not only to validate our purification method but also to compare the results with those of the applied LOH analysis. In the small series of described cases described herein, we identified 2 cases (cases C11 and...
The combined application of flow cytometry and flow cytometric sorting is an essential advantage when studying the various molecular aberrations in heterogeneous tumors such as lymphomas but can also be used for studying other tumors with a high content of nonneoplastic cells.

T13) with aneusomy of chromosome 6 and 1 case with possible duplication of a part of the HLA region (case T26).

From the Departments of Pathology, 1Leiden University Medical Center, Leiden; 2Atrium Medical Center Heerlen, Heerlen; and 3University Hospital Groningen, Groningen, the Netherlands.

Supported by grant RUL99-1997 from the Dutch Cancer Society, Amsterdam, the Netherlands.

Address reprint requests to Ms Jordanova: Dept of Pathology, L1Q, P1-40, Leiden University Medical Center, Albinusdreef 2, 2333 ZA, Leiden, the Netherlands.
Acknowledgments: We thank Eric Noteboom and Anita Pfauth, Netherlands Cancer Institute, Amsterdam, for excellent technical assistance with the flow cytometric sorting procedure; Leendert Looijenga, MD, PhD, Josephine Nefkens Institute, Rotterdam, for the lymphoma material provided; and Marius Nap, MD, PhD, Atrium Medical Center, Heerlen, for critically reading the manuscript.

References


