Minimal Disease Detection and Confirmation in Hematologic Malignancies: Combining Cell Sorting with Clonality Profiling

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Background: In this study we demonstrate the technical application of flow cytometry and cell sorting combined with gene-rearrangement clonality profiling to detect and confirm minimal disease in 2 leukemia and 2 lymphoma cases.

Methods: Specimens with low percentages (0.05%–5%) of abnormal lymphoid populations were identified by flow cytometry. The abnormal lymphoid populations were sorted by flow cytometry, and the purified tumor populations along with unsorted fractions were subsequently analyzed for the presence of clonal gene rearrangements by PCR and fluorescence-based capillary electrophoresis fragment analysis.

Results: In 3 cases, distinct clonality profiles could be detected in the purified tumor cell fraction, and suspicious amplicons of identical sizes were detected among the polyclonal backgrounds in the unsorted specimens. For 1 patient, a monoclonal signal was detected in the sorted tumor cell fraction but not in the unseparated bone marrow specimen containing 0.05% abnormal lymphoblasts. A subsequent bone marrow specimen containing 4.8% recurring leukemia cells tested positive with a clonality profile that matched the previous profile in the sorted cell population.

Conclusions: The described method integrating 2 technologies allows genotypic confirmation of an aberrant population detected by immunophenotype to increase diagnostic certainty. This strategy provides a sensitive tool for disease monitoring without the need for patient-specific primer design and assay optimization required for quantitative PCR analysis.

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Minimal disease may be the product of therapy (residual disease) or may simply be the early detection of a minor malignant clone at diagnosis. Several studies have shown that quantitative detection of minimal residual disease (MRD)4 in lymphoid malignancies predicts the clinical outcome (1–12). Monitoring treatment response by quantification of the tumor load is crucial to assess the risk of relapse and to identify those patients who may benefit from therapy reduction or intensification, reduction of immunosuppression for graft-vs-leukemia effect after stem cell transplantation, or adoptive T-cell therapy (13). Minimal disease may also be encountered in primary diagnostic situations, for example, low concentrations of monoclonal B cells in patients with cytopenias suspicious for myelodysplastic syndromes (14).

Immunoglobulin and T-cell receptor (TCR) gene rearrangements are frequently used as targets in PCR-based MRD studies (15–18). These rearrangements can be considered as “fingerprints” for lymphoid cells, because each clone has its own deletions and random insertions of nucleotides at the junction sites of the gene segments. A clonal leukemic cell population of lymphoid origin can be detected by the presence of a strong signal for a single gene rearrangement of a specific size after multiplex PCR amplification, whereas a polyclonal lymphocyte population leads to uniform gaussian distribution of amplicons.

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4 Nonstandard abbreviations: TCR, T-cell receptor; TCRG, T-cell receptor γ chain; IgH, immunoglobulin heavy chain; Mah, monoclonal antibody; LGL, large granular lymphocytic leukemia; and FR, framework region.
To use these gene rearrangements for MRD analysis by real-time PCR, patient-specific assays must be created after sequencing of the gene-rearrangement amplicon. Several studies have compared the lower limit of detection in MRD for flow cytometry and molecular approaches (19–23). Flow cytometer–based immunophenotyping provides a rapid and sensitive method for detecting up to 1 leukemic cell in 10^4 healthy cells. Molecular analysis of gene rearrangements can routinely detect a minimum of 1 monoclonal B cell in 1000 healthy cells using immunoglobulin heavy-chain (IgH) multiplex assays and 1 monoclonal T cell in 100 healthy cells using TCRγ primer sets. Patient-specific real-time quantitative PCR assays can be established for immunoglobulin/TCR gene rearrangements with detection limits ranging from 0.01% to 0.001%, but assay setup is currently too time-consuming for a routine clinical application. In this study we demonstrate a 2-step technique for detecting and confirming residual disease by integrating phenotype analysis using standardized flow cytometry panels, cell sorting, and subsequent genotype analysis by multiplex gene-rearrangement PCR.

Materials and Methods

FLOW CYTOMETRY AND CELL SORTING
Antibody combinations, their sources, and the detailed procedures for flow cytometric analyses have been described previously (24). Briefly, staining for flow cytometric analysis was performed with a 20-min incubation at ambient temperature with the titrated monoclonal antibodies (Mabs) of interest followed by erythrocyte lysis by use of an ammonium chloride solution. Cells were then fixed with 10 mL/L paraformaldehyde and analyzed on a FACSCalibur flow cytometer (BD Biosciences). Data analysis using CD45 gating was performed with WinList (Verity Software House) (25). Cell sorting of viable cells was performed on a FACS Vantage SE Cell Sorter (BD Biosciences) with selected Mab combinations to target the cell populations of interest as described in the Results and Discussion section. Cell sorting rates from 1000 to 3500 cells/s were used.

PCR STUDIES OF GENE REARRANGEMENTS
Genomic DNA was isolated from sorted and unsorted cell samples by use of the QIAamp® DNA Mini Kit (Qiagen) according to the manufacturer’s instructions. For clonal B-cell gene-rearrangement detection, DNA was amplified using the Biomed-2 (26) primer sets for the IgH framework regions (FRs) 1–3 according to the manufacturer’s instructions (InVivoScribe Technologies). To identify T-cell clonality, the TCRγ gene-rearrangement assay (InVivoScribe Technologies) was used according to the instructions. Monoclonal, polyclonal, and no-template control samples were included for each PCR assay. PCR amplicons were analyzed by fluorescence detection using the ABI310 capillary electrophoresis sequencer and the ABI Prism® GeneScan® Analysis software.

Results and Discussion

In this report, we demonstrate the feasibility of combining cell sorting with clonality profiling to effectively improve the sensitivity of disease detection and to provide independent confirmation of tumor detection without the need for patient-specific assay designs. Bone marrow aspirates from 3 patients with small abnormal B-lymphoid populations and 1 peripheral blood specimen with a small aberrant T-cell population, as detected by flow cytometry, were analyzed by IgH or TCRγ chain (TCRG) gene-rearrangement PCR with and without cell purification to illustrate the utility of relating aberrant phenotypes to a specific genotype for several clinical applications.

STAGING LYMPHOMA

Sampling of bone marrow for disease staging to assess the dissemination of lymphoma is a common application of minimal disease detection using flow cytometry. Often the diagnosis is made on a lymph node biopsy that is never sent for confirmation by flow cytometry. The quandary is detecting a small population of abnormal cells by flow cytometry and correlating these results with the morphologic diagnosis. Without a phenotypic fingerprint, the detection of abnormal cells is based on the difference from healthy cells (7, 27). The specificity and sensitivity of the assay depend on the reagents, the instrument sensitivity, the frequency of abnormal cells, the frequency of healthy counterparts, and the relative differences between the healthy and abnormal cells. These factors interact to increase or decrease the level of confidence in deciding whether the specimen contains a tumor. This is especially critical when treatment will be based on the results of the test.

An example of this type of situation was observed when a bone marrow specimen from a 53-year-old male, recently diagnosed with follicular lymphoma, was submitted for staging by flow cytometry. The original diagnosis was based on morphology and immunocytochemistry on a lymph node biopsy. The original diagnostic specimen was not available for assessment of tumor phenotype or genotype. The flow cytometry antibody panel, which included CD45, HLA-DR, CD19, FMC-7, CD20, CD10, CD5, CD22, CD25, CD23, and immunoglobulin κ and λ light chains, showed a slight increase in CD10 and CD20 staining intensity in cells phenotypically identical to stage III immature B-lymphoid cells (27). A suggestive increase in λ staining was detected in a small abnormal lymphoid population (0.6%) with low CD19 expression. Reprocessing of the specimen correlating CD10 with surface κ and λ also suggested an increase in λ staining; however, this difference was not definitive because a majority of the B-cell population (4.6% of nonerythroid cells) was polyclonal.

We therefore used B-cell gene-rearrangement studies in combination with flow cytometry-based cell sorting to confirm monoclonality in that specific population of cells (Fig. 1). We extracted genomic DNA from 40 000 cells...
purified by flow cytometry for CD10 and CD45 expression (Fig. 1A) and from 45,000 purified CD45-positive lymphocytes that did not express CD10. We then studied the isolated DNA by genotype analysis using multiplex gene-rearrangement PCR. Monoclonal peaks were detected at 346 bp for IgH FR1 and at 281 bp for FR2 in the unsorted and in the CD10-positive sorted cell fraction (Fig. 1, B and C). The sorted control cell fraction (CD10−/CD45+) containing healthy developing B and T cells showed polyclonal amplicon distribution for all 3 immunoglobulin framework regions (data not shown). These data demonstrate that the abnormalities detected by flow cytometry and by gene-rearrangement studies were identifiable in the same cell population. Cell purification allows for independent phenotype and genotype studies to be performed on exactly the same aberrant cells and not just correlative to the entire specimen, thus increasing the specificity of both techniques.

RESIDUAL DISEASE MONITORING

The detection of low numbers of tumor cells after therapy is the most frequent use of minimal disease detection. Standardized panels of monoclonal antibodies can be used for residual disease detection, circumventing the requirement for a diagnostic specimen or the detection of a clonal population that changes phenotype (7, 27, 28). This approach is useful in a bone marrow transplantation setting, in which the patient is often first encountered when in remission. The detection of MRD is illustrated in a specimen from a 19-year-old patient after hematopoietic stem cell transplantation for precursor B-lineage acute lymphoblastic leukemia. The patient had relapsed after the transplant and was given chemotherapy to induce remission. A bone marrow aspirate was obtained to assess remission status. After analysis of the bone marrow, 0.05% abnormal lymphoblasts were detected by flow cytometry that were characterized by HLA-DR, bright CD10, dim CD19, and bright CD34 expression, but which lacked CD45 expression. No monoclonal or polyclonal signals were detected by the IgH gene-rearrangement assay in this bone marrow specimen because of the presence of very few healthy B cells (0.02%; data not shown). The small abnormal lymphoblast population was sorted by use of a CD10+/CD45− gate (Fig. 2A), and 800 purified cells were analyzed for B-cell clonality by PCR. The sorted tumor cell population had a monoclonal peak profile with amplicons at 115 and 164 bp for IgH FR3, 254 bp for FR2, and 314 and 360 bp for FR2 (data not shown). The combination of aberrant phenotype and monoclonal cell population demonstrated that the tumor was still detectable at 0.05%.

A follow-up bone marrow specimen was received 5 weeks later, and flow cytometry revealed increased abnormal lymphoblasts at 4.8% with an identical phenotype as detected before, clearly indicating relapse. B-Cell gene-rearrangement analysis of this unseparated bone marrow specimen produced monoclonal amplicons identical in size to the clonality profile detected in the previous sorted cell fraction (data not shown). After additional chemotherapy, an additional bone marrow aspirate obtained 4...
weeks later contained 0.3% residual abnormal lymphoblasts by flow cytometry analysis. B-Cell gene-rearrangement analysis of the unseparated bone marrow did not produce a distinct monoclonal profile because of the low percentage of tumor cells (Fig. 2B). However, the B-cell clonality profile of the CD10-positive sorted cell population again revealed clonal peak sizes identical to previous results (Fig. 2C).

B-Cell gene-rearrangement PCR alone can be used to assess and monitor clonality if the suspected malignant population is present at a concentration of ~1%. To increase the sensitivity for MRD monitoring, the use of allele-specific primers in combination with germline (Jg) primers and (Jg) TaqMan probes have been shown to be useful tools that can detect tumor cells in concentrations as low as 0.01% (1 leukemic cell in a background of 1 × 10^4 healthy cells) to 0.001% (1 leukemic cell in a background of 1 × 10^5 healthy cells) (15–17, 29–31). However, in many cases those detection limits cannot be reached because of nonspecific amplification of gene rearrangements in the healthy lymphocytes. After treatment, the background of healthy B and T cells may be particularly high, increasing the detection limit even further. In addition to the cumbersome setup of patient-specific assays, several gene-rearrangement loci should be used simultaneously as PCR targets because single rearrangements are unstable and can be lost during clonal transformation and after disease relapse because of continuing gene rear-

Fig. 2. A small abnormal population of cells was detected by flow cytometry in the bone marrow from a patient with precursor B-cell acute lymphoblastic leukemia after hematopoietic stem cell transplantation.

(A), the cell fraction in the rectangle was sorted by use of a CD10+/CD45− gate for B-cell gene-rearrangement studies. (B), molecular analysis of unseparated bone marrow showed no distinct monoclonality profile. (C), the sorted tumor cell population demonstrated a monoclonal peak profile with amplicons at 115 and 164 bp for IgH FR3, 254 bp for FR2, and 314 and 360 bp for FR1. The identical monoclonality profile was found in 3 monitoring specimens with MRD from this patient (see Results and Discussion). RFU, relative fluorescence units.
rangements or further gene deletions. In particular, for malignancies demonstrating oligoclonality with multiple subclones present at diagnosis, the likelihood of losing a PCR target during follow-up is increased substantially (18). In this study, we combined routine immunophenotyping with cell purification and subsequent gene-rearrangement studies by multifluorescent PCR and capillary electrophoresis analysis to confirm the presence of a monoclonal leukemic cell population and to lower the assay detection limit. Suspicious cell populations present in concentrations as low as 0.01% can be identified and purified by flow cytometry. Subsequent gene-rearrangement analysis can confirm the identity of putative monoclonal peaks with reference to the unsorted specimen and/or to the original clone from a diagnostic specimen or paraffin-embedded tumor biopsy. The combination of the 2 technologies allows the identification of a clonogenic neoplastic cell population that would be undetectable or inconclusive by conventional analysis. For minimal disease monitoring, the amplicon size of the clonal gene rearrangement, known from a previous diagnostic marrow aspirate or paraffin-embedded biopsy specimen, becomes the tumor-specific marker without the need to develop patient-specific DNA primers, probes, or antibody panels. This approach could also be used to demonstrate that a suspicious phenotype is not monoclonal and/or recurrent disease, thus preventing potential additional chemotherapy for the patient.

Fig. 3. Bone marrow aspirate from a patient with hemolytic anemia and a final diagnosis of small lymphocytic lymphoma revealed a small population of mature B-lymphoid cells that coexpressed CD5 and CD19. (A), the CD5+ and CD19+ cells in the oval were sorted for B-cell gene-rearrangement studies. (B), molecular analysis of the unseparated bone marrow specimen detected a putative monoclonal peak at 320 bp for IgH FR1 and polyclonal amplicon distribution for FR2 and FR3. (C), a single distinct monoclonal peak at 320 bp for FR1 was detected in the purified CD5+ and CD19+ cell fraction. RFU, relative fluorescence units.
In addition to MRD monitoring, cell sorting in combination with clonality profiling can also provide valuable confirmatory data in primary diagnostic specimens with low proportions of neoplastic cells. Patients presenting with anemia and/or pancytopenia often provide a difficult diagnostic dilemma. In addition to multiple nonneoplastic etiologies, clonal processes in myeloid, T, or B cells can cause suppression of hematopoiesis (24, 32). The abnormal cell population may constitute a minor proportion of the specimen yet can influence the production of cells of multiple lineages.

B cells
We obtained a diagnostic bone marrow aspirate from a 66-year-old female with a clinical history of autoimmune hemolytic anemia and cold agglutinins. Flow cytometry findings revealed a small abnormal B-lymphoid population at 0.6% of the total nonerythroid cells positive for HLA-DR, CD38, CD19, and bright CD20. Healthy B-lymphoid cells were more frequent, at 6.1% of the nonerythroid cells. Four-color flow cytometric analyses of CD5, CD19, and CD45 combined with immunoglobulin light chain expression demonstrated a predominance of surface immunoglobulin κ light chain expressed on the CD19+/CD5+/bright CD45+ cells. However, there still was a background of cells expressing λ light chain. Because this was the primary diagnostic specimen for this patient, the identity of an aberrant B-cell population required confirmation by other techniques. Gene-rearrangement analysis of the bone marrow specimen detected a suspicious but not a definitive monoclonal peak at 320 bp for IgH FR1 (Fig. 3B). Cells positive for CD5 and CD19 expression were sorted by flow cytometry with subsequent DNA extraction and analysis for B-cell gene rearrangements (Fig. 3A). A distinct monoclonal peak at 320 bp for FR1 was detected in the purified tumor cell fraction (Fig. 3C), confirming monoclonality of the small aberrant B-cell population.

T cells
T-Cell gene-rearrangement studies are less sensitive and specific in detecting small populations of cells compared with B-cell gene studies because of primer cross-reactivity with polyclonal background and the high frequency of benign clonal T-cell expansions, particularly in peripheral blood (16, 26, 33–35). Cell purification based on phenotype can solidify the identification and characterization of abnormal T-cell populations. For a 72-year-old female with a clinical history of anemia, flow cytometry revealed 5% abnormal T cells in the peripheral blood specimen with an immunophenotype consistent with large granular lymphocytic leukemia (LGL). The cells were small and expressed increased CD3, decreased CD5, and CD56 as well as normal concentrations of the T-cell antigens CD2 and CD7, but they did not express CD4, CD8, CD1a, CD25, or CD30. The abnormal cells expressed TCRγ/δ, whereas the healthy T cells expressed TCRα/β.

Total peripheral blood was analyzed by T-cell gene-
rearrangement PCR, and a putative monoclonal peak was detected at 245 bp (Fig. 4B). Using the CD56+ and CD3+ gates, we purified 20,000 cells of the abnormal T-cell population for comparison with the healthy T cells that did not express CD56 (Fig. 4A). Subsequent analysis for TCRγ gene rearrangement demonstrated a single monoclonal peak at 245 bp, matching the putative peak in the unsorted specimen (Fig. 4C). Polyclonal amplicon distribution was detected in the internal control cell fraction purified by a CD56+/ CD3+ gate (data not shown).

Although reactivity with discrete Mabs is suggestive of expansion of a particular granular lymphocyte population in LGL diagnosis, it is important to demonstrate clonality (32, 36, 37). The interpretation of putative monoclonal peaks can be particularly difficult in specimens with small T-cell populations. Although detection limits reported for TCRγ gene-rearrangement analysis have ranged from 1% to 0.1% (1 leukemic cell in backgrounds of 100 to 1000 healthy cells) (38), in our experience the detection limit of the TCRγ PCR assay is highly variable from patient to patient, depending on the polyclonal background of the specific gene rearrangement. The TCRγ gene-rearrangement assay can indeed detect tumor cells at 1% and lower, but for a subset of tumor specimens, strong monoclonal signatures are achieved only if the abnormal cells are present at concentrations of 5%–10% or greater. Moreover, if the size of the patient-specific gene-rearrangement signal is unknown, the interpretation of small putative monoclonal peaks can be difficult. These findings are in agreement with the literature reporting that the sensitivities for TCR genes as PCR targets are dependent on the frequencies of comparable gene rearrangements in healthy cells (33). In particular, TCRγ gene rearrangements are known for their limited sensitivities because of the limited size of TCRγ junctional regions and the abundant background of polyclonal TCRγ gene rearrangements in healthy T cells (15, 33). TCRγ gene rearrangements occur on both alleles in virtually all CD3+ lymphocytes and show limited combinatorial and junctional diversity. On the other hand, TCRγ gene rearrangements are known for their high stability from diagnosis to relapse in T-cell acute lymphoblastic leukemia because they are mostly end-stage rearrangements (34). Overinterpretation of small suggestive peaks must be avoided because oligoclonal/clonal T-cell expansions can also be found in healthy individuals. So-called “canonical” TCRγ rearrangements resulting from accumulation of TCRγ6+ T lymphocytes can be found particularly in peripheral blood and increase in frequency with age (26, 35, 39). Reactive oligoclonal populations can also occur after transplantation that represent repopulation of the bone marrow and cannot be interpreted as reoccurrence of malignancy without reference to the original clone. Therefore, flow cytometry cell sorting can become a helpful asset to link abnormal phenotype with monoclonality and the converse.

In summary, this study demonstrates the technical application of standardized flow cytometry panels to identify and to purify minor abnormal B- and T-cell populations for further molecular analysis. Four case studies outline the usefulness of this approach in minimal disease confirmation in staging, monitoring, and diagnostic settings; however, validation studies with larger numbers of patients are still required and are currently pending.

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References

13. Bradfield SM, Radich JP, Loken MR. Graft-versus-leukemia effect...


