An accurate and rapid flow cytometric diagnosis of BCR-ABL positive acute lymphoblastic leukemia

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ABSTRACT

Tyrosine kinase inhibitors have profoundly modified the treatment and prognosis of chronic myeloid leukemia and Ph+ acute lymphoblastic leukemia. A rapid and accurate detection of the BCR-ABL fusion protein is paramount today for an optimal management of Ph+ acute lymphoblastic leukemia. We have utilized a recently described and commercialized immunoassay that identifies qualitatively the presence of the BCR-ABL protein in leukemic cell lysates. The BCR-ABL fusion protein is captured and detected by a cytometric bead assay and analyzed by flow cytometry. The assay was applied to 101 primary patient samples (94 acute leukemias and 7 chronic myeloid leukemia blast crisis) and the results of the immunoassay were concordant with those obtained by conventional molecular techniques. The method proved reliable, reproducible, of simple execution and it was successfully completed within four hours. This flow cytometric immunoassay has important implications for perfecting the management of Ph+ acute lymphoblastic leukemia patients worldwide.

Key words: flow cytometric diagnosis, acute lymphoblastic leukemia.

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Introduction

Acute lymphoblastic leukemia (ALL) is the most common malignancy in children, but it also affects adults with an incidence that progressively increases with age.[1-3] Different genetic abnormalities are found in ALL, the most frequent in adult cases being the Philadelphia (Ph) chromosome. Broad and integrated cytogenetic-genetic analyses have allowed prognostic subgroups in adult ALL to be defined.[4] Both in children and in adults the presence of the Ph chromosome is associated with a very unfavorable prognosis.[1-5] The Ph chromosome is created through the translocation of a section of human chromosome 9 that contains the Abelson (ABL) kinase domain, with a specific breakpoint cluster region (BCR) on chromosome 22.[6,7] The resulting fusion gene *BCR-ABL* encodes for an abnormal, non-membrane-bound oncoprotein. The mechanisms of action of the BCR-ABL fusion protein are still not fully understood, but the oncoprotein is a constitutively active tyrosine kinase that perturbs numerous signal transduction pathways resulting in uncontrolled cell proliferation, reduced apoptosis and impaired cell adhesion. Three fusion proteins of different sizes may be produced (p190, p210 and p230) depending on the site of the breakpoint within the *BCR* gene. These alterations are present in 2-5% of children, in 25-30% of adults and in about 40% of the older ALL patients.

The development of inhibitors directed specifically against the BCR-ABL tyrosine kinase (TKI) has opened innovative therapeutic avenues that have profoundly impacted on the management of the diseases harboring this genetic abnormality. Indeed, TKI have modified the natural history of chronic myeloid leukemia (CML)[8,9] and their use has more recently been extended to the management of Ph+ ALL patients.[10,11] In fact, TKI, in combination with chemotherapy or alone, is nowadays utilized upfront as first line therapy for Ph+ ALL.[12-15] These recent developments highlight the need for a rapid and reliable identification of this genetic lesion in patients with ALL at presentation, for whom molecular methods, by real time quantitative polymerase chain reaction (RQ-PCR) amplification, and cytogenetic analyses, by conventional karyotype and fluorescence in situ hybridization (FISH), represent the methods currently used for the identification of both the *BCR-ABL* gene transcripts and the *BCR-ABL* genes. These techniques, however, require the availability of specialized laboratories and are often time consuming. On the basis of the above considerations, in the present study we have tested a recently developed flow cytometric assay designed to detect the BCR-ABL fusion protein on primary acute leukemia samples[16] and have determined the applicability, reliability, specificity and rapidity of this method.

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Design and Methods

Patients and leukemia characterization

The presence of the BCR-ABL protein was investigated on fresh bone marrow or peripheral blood samples of 101 cases (91 adults and 10 children) with acute leukemia referred to our Institution, between April 2008 and November 2008.

Fifty-nine patients were males and 42 females; the median age was 45 years (range 1-81). The diagnosis of each leukemia subtype was established according to morphological, cytochemical and immunological criteria according to the French-American-British (FAB) and World Health Organization (WHO) classifications. Whole bone marrow or peripheral blood cells were stained with various combinations of fluorescein isothiocyanate (FITC), phycoerythrin (PE)-, peridin-clorophyll protein (PerCP) or phycoerythtin cyanin 5 (PC5)- and allophycocyanin (APC)-labeled monoclonal antibodies against the following antigens: CD1a, CD10 (Dako, Glostrup, Denmark), CD2, CD3, CD4, CD5, CD8, CD11b, CD13, CD14, CD15, CD19, CD20, CD34, CD38, CD45, CD56 (BD, Biosciences, San Jose, CA), CD7, CD33, CD65, CD66c, CD117 (Immunotech Coulter Company, Marseille, France), CD133 (Miltenyi Biotec, Bergisch Gladbach, Germany). Other antibodies were used to identify cytoplasmic antigens MPO, CD3, CD79a, Dako, and immunoglobulin M-heavy chain (cyIgM), (Southern Biotech, Birmingham, AL) and nuclear terminal deoxynucleotidyl transferase (TdT) (Dako).

We investigated 89 acute leukemia cases at the time of diagnosis. Based on the immunophenotypic profile, these cases could be further subdivided as follows: B-lineage ALL in 53 cases (8 pro-B, 38 B-common and 7 pre-B), T-ALL in 5, acute myeloid leukemia (AML) in 30 and acute biphenotypic leukemia in one case. We also studied 5 relapsed cases (one T-ALL, 3 B-common ALL and one AML) and 7 cases of CML blast crisis (4 B and 3 myeloid).

All patients' samples were also studied in our molecular biology laboratory in order to identify the presence of fusion transcripts using a multiplex reverse transcription polymerase chain reaction (RT-PCR).[17] Within the ALL cases the analysis identified the following fusion transcripts: BCR-ABL in 32 cases (p190 in 19, p210 in 4 and p190/p210 in 9 cases), MLL-AF4 in 3 cases, MLL-ENL in 2 cases, E2A-PBX1 in 2 cases and SIL-TAL1 in one case. Among the AML cases, 6 cases of PML-RARα and one case of NUMA-RARα were identified.

Analysis of the BCR-ABL protein

For the identification of the presence of the BCR-ABL fusion protein, we utilized the BCR-ABL Protein Kit (BD Biosciences). The Cytometric Bead Assay (CBA) consists of an immunoassay that identifies qualitatively the presence of the BCR-ABL protein in the lysates of the leukemic cell population. By lysis of the leukemic cells, the fusion proteins are released and they are recognized by an anti-BCR antibody coupled to a bead and a PE-labeled anti-ABL antibody.[16] Lysates from normal peripheral leukocytes (WBC) and from the K562 cell line were used as negative and positive controls, respectively.

According to the manufacturer's instructions, 1-2 mL of whole blood/marrow specimens containing 25×10^6 cells were incubated with 50 ml of a 1X stock of the BD Pharm Lyse lysing buffer (BD Biosciences), for ten minutes at room temperature with occasional mixing to lyse red blood cells. Cells were washed twice by adding PBS with 5% FBS. Subsequently, at least 250 µL of the Pretreatment Buffer obtained by diluting the 1X stock of BCR-ABL Pretreatment A (BD Biosciences) and the 1X stock of BCR-ABL Pretreatment B (BD Biosciences) were added to each sample and controls; samples were then incubated on ice for ten minutes and washed once by adding PBS with 5% FBS. Thereafter, samples and controls were incubated for 15 minutes with 100 µL of the 1X stock of the BD Lysate Treatment Reagent (BD Biosciences) and then centrifuged at 20,000g for ten minutes at 4°C. Fifty µL of the cell lysates, from both samples and controls, were incubated for two hours with 50 µL of an anti-BCR antibody coupled to a bead (BD Biosciences) and 50 µL of the PE-labeled anti-ABL antibody (BD Biosciences). After washing with the CBA Wash Buffer (BD Biosciences Pharmigen), samples were resuspended in 300 µL of the CBA Wash Buffer and acquired on a flow cytometer (FACSCanto, Becton Dickinson) using the FACSDiva software version 6.0 (all from BD Biosciences), after FACSCanto setting using the Cytometer Setup and Tracking (CS&T) Bead system according to the manufacturer's guidelines (BD Biosciences). On average, there are 6,000 beads/50 µL of beads suspension. The absence of the BCR-ABL fusion protein was defined utilizing the mean ± 2SD of PE MFI of normal peripheral leukocytes.

Results and Discussion

Thirty of the 101 (29.7%) cases studied proved positive for the BCR-ABL fusion protein, 24/30 collected from the bone marrow and 6/30 from the peripheral blood. In these samples, the median number of leukemic cells was 17.6×10^6 (range 0.8-23.7). Twenty-three were affected by B-lineage ALL and 7 by blast crisis of CML. All transcripts (p190, p210, p190/210) were recognized by the CBA; none of the cases analyzed carried the p230 transcript (Table 1). The fusion transcript was detected by RQ-PCR in all CBA positive patients. Two cases negative by CBA proved positive by RQ-PCR. However, these latter samples, collected from 2 B-common ALL patients, contained only 5.9×10^5 and 5.1×10^5 leukemic cells (2.3% and 2%, respectively, of the total population) at the time of the analysis. This was due to the fact that both patients were on steroid treatment. We have thus shown a degree of correlation of 100%, between flow cytometry identification of the BCR-ABL fusion protein and molecular biology identification of the fusion transcript, provided that the number of leukemic cells in the preparations to be analyzed was greater than 8×10^5.

We have repeated the flow cytometric determination of the fusion protein in 3 positive patients after 48 or 96 h at room temperature and have found a positive signal, even if less intense compared to the signal obtained from fresh cells processed within 24 h. Similar results have been achieved on leukemic cell lysates stored at -80°C and thawed after four, 18 and 22 days.

With regard to the group of the 71 (70.3%) patients with a negative CBA test, 57/71 collected from the bone marrow and 14/71 from the peripheral blood, the median number of leukemic cells was 17.5×10^6 (range 0.51-24.5). In these patients the diagnosis, documented by immunophenotypic analysis, was: B-lineage ALL in 32 cases, T-ALL in 5, AML in 30, acute biphenotypic leukemia in one and relapse in 3 (B-common ALL in one, T-ALL in one and AML in one case) (Table 1). Among the cases with B-lineage ALL, 5 had pro-B ALL, 3 of which showed the MLL-AF4 transcript and 2 the MLL-ENL transcript; among the AML cases there were 6 cases with PML-RARα and one with the NUMA-RARα transcript.

Taken together, these results show the specificity of this flow cytometric assay for the identification of the BCR-ABL fusion protein (Figure 1). In fact, we have documented an absolute correlation between the presence or absence of the BCR-ABL protein evaluated using the BCR-ABL protein kit and the positivity or negativity of the transcript assessed according to conventional molecular techniques. In our laboratory, this dosage has been shown to be reliable, reproducible, of simple execution and it was always successfully completed within a maximum of four hours from the marrow or blood collection on 25×10^6 total cells containing at least 10% of leukemic cells, as in the manufacturer's instructions. Moreover, the result obtained from worked fresh cells after 48 or 96 h and thawed leukemic cells at -80°C indicate that samples

Table 1. Results of the flow cytometric immunobead assay (CBA) compared to the *BCR/ABL* transcript detection by PCR.

Samples tested (n=101)	BCR-ABL CBA		BCR-ABL PCR assay			
	negative	positive	negative	p190	p210	p190/p210
B-lineage ALL (n=56)						
B-common ALL (n=38)	21*	17	19*	14	2	3
Pre-B ALL (n=7)	5	2	5	0	1	1
Pro-B ALL (n=8)	6	2	6	2	0	0
B-common relapse (n=3)	1	2	1	2	0	0
T-ALL (n=6)						
Diagnosis (n=5)	5	0	5	0	0	0
Relapse (n=1)	1	0	1	0	0	0
AML (n=31)						
Diagnosis (n=30)	30	0	30	0	0	0
Relapse (n=1)	1	0	1	0	0	0
Biphenotypic acute	1	0	1	0	0	0
leukemia (n=1)						
Blast crisis of CML (n=7)						
B-lineage ALL (n=4)	0	4	0	0	1	3
Myeloid (n=3)	0	3	0	1	0	2

**The two samples negative with the CBA assay contained only 5.9×10^5 and 5.1×10^5 leukemic cells and were drawn from patients who were on steroid treatment.*

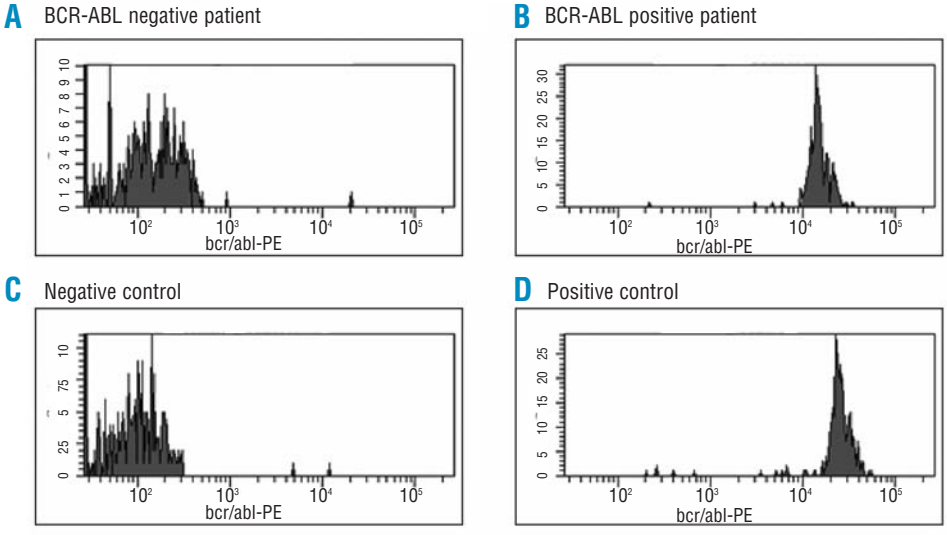

Figure 1. Detection of the bcr-abl fusion protein with the flow cytometric immunobead assay. (A) Cell lysate from a patient negative for the BCR-ABL transcript but positive for the t(15;17) transcript. (B) Cell lysate from a patient positive for the BCR-ABL transcript. (C) Cell lysate from normal peripheral leukocytes. (D) Cell lysate from the BCR-ABL positive K562 cell line.

can be successfully processed also within a few days from being sent or arrival in the laboratory, as well as on lysates stored at -80°C for a number of days, without important differences in comparison to the results obtained with fresh cells processed within 24 h.

These results are in agreement with those recently described by Weerkamp *et al.*,[16] with the exception of the sensitivity of the test that, in our experience on primary leukemic samples, could not detect the fusion protein in 2 steroid-treated patients due to the very low leukemic cell counts at the time of the analysis. It should, however, be noted that 2 other steroid-treated patients with higher leukemic cell counts proved positive. Further investigations on the effect of steroid treatment on patients' leukemic cell counts, *BCR-ABL* transcript and BCR-ABL protein are warranted to better understand the biology of the disease. From a practical point of view, virtually no ALL patient at diagnosis and untreated has very low numbers of marrow or blood leukemic cells.

The availability of a method capable of highlighting the presence of the BCR-ABL protein has important implications because it can document the effective transduction of the molecular transcript. It allows rapid identification of the presence of the BCR-ABL protein (p190, p210 and p190/p210) through a flow cytometric analysis and thus to reliably offer proteomic information to investigators researching targeted anti-tyrosine kinase treatment. This identification can translate into the timely implementation of a targeted therapeutic strategy. This is currently a primary unmet need in many geographical regions in light of the recent results with 1st and 2nd generation TKI inhibitors alone as first line treatment for Ph+ adult ALL patients.[13-15] Indeed all patients (with no upper age limit) can be induced into a complete hematologic remission with an oral TKI, no chemotherapy and partly at home. This has a profund impact on the overall approach to this most unfavorable condition, the worst in hematology prior to the advent of TKI, but requires that the presence of the BCR-ABL fusion protein is accurately and rapidly detected in all newly diagnosed (and relapsed) ALL patients, irrespective of age. Since PCR technologies are not available in many areas of the world, the possibility of utilizing a rapid, simple and reliable flow cytometry technique opens the way to offering a much required targeted diagnostic and therapeutic approach for an optimal management of Ph+ ALL both in children and in adults worldwide.

Authorship and Disclosures

SR and RF were the principal investigators and took the primary responsibility for the paper; SR, MSDP, SI, LE and DD performed the laboratory work for this study; HW performed the standardization of the BCR-ABL protein dosage; AV managed patients and took care of sample collection; AG co-ordinated the research; SR, AG and RF wrote the paper.

HW is currently employed at BD Biosciences, San Jose, Ca, USA. The other authors reported no potential conflicts of interest.

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