

# Comparison of Multidimensional Flow Cytometry With Standard Morphology for Evaluation of Early Marrow Response in Pediatric Acute Lymphoblastic Leukemia

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**Purpose:** We compared multidimensional flow cytometry (MDF) with morphology in evaluating early marrow response to induction chemotherapy in pediatric ALL.

**Methods:** Chemotherapy response was determined by standard morphology or by MDF assessed by residual leukemic cell percentage remaining in the marrow on days 7, 14, and 28 of induction. Bone marrow response was classified as M3 (>25% leukemic blasts) or M1/M2 ( $\leq$ 25% leukemic blasts). Multidimensional flow cytometry evaluation was compared with that of standard morphology. Available day-7 and day-14 marrow slides were also reevaluated by a single pathologist without patients' clinical information.

**Results:** Of 46 day-7 specimens, eight (17%) had discordant MDF and morphology results ( $P < 0.001$ ), including six classified as M3 by morphology but were M1/M2 by MDF, and two were classified as M3 by MDF but were M1/M2 by morphology. Of 24 day-14 bone marrow specimens, five (20.5%) were discordant ( $P < 0.001$ ), including two classified as M3 by morphology but were M1/M2 by MDF, and three were classified as M3 by MDF but were M1/M2 by morphology. Reevaluation of the blinded day-7 and day-14 marrow slides yielded discordance between repeated pathology readings of 11% ( $P < 0.001$ ) and 6% ( $P = 0.04$ ), respectively.

**Conclusion:** Our data show significant discordance between the morphologic and MDF evaluation of early marrow response. Early response to therapy is a significant prognostic indicator in pediatric acute lymphoblastic leukemia and is used to alter subsequent treatment; thus, precise assessment of response is important. A larger comparison of MDF with morphology for the evaluation of early response, including correlation with clinical outcome, is warranted.

**Key Words:** Acute lymphoblastic leukemia—Children—Multidimensional flow cytometry—Morphology.

Childhood acute lymphoblastic leukemia (ALL) is the single most common childhood malignancy. With an incidence of more than 30 cases per one million children younger than 15 years of age per year, ALL comprises almost one-fourth of all pediatric cancer (1). Multiagent chemotherapy has significantly improved survival for childhood ALL (2). However, the relapse rate with current therapies remains at 20% to 30% (3). Relapsed ALL continues to have unacceptably poor outcome, especially in bone marrow relapse (4,5). Risk-based therapy, directed by early identification of patients with unfavorable clinical and laboratory features, has improved outcome for patients at high-risk while avoiding chemotherapy-related morbidity for the patients at low-risk.

Rapidity of response to chemotherapy is a significant prognostic indicator in numerous malignancies (6–9). In childhood ALL, early reduction in leukemic load after initiation of chemotherapy is a major prognostic factor for survival. Specifically, reduction in peripheral leukemic blasts has prognostic significance and has been a criterion for risk-directed therapy (10). European cooperative groups have stratified patients into standard-risk and high-risk categories based on their response to 1 week of single agent therapy with prednisone (11,12). Patients with rapid response to prednisone (<1,000 peripheral blasts/ $\mu$ L) have a significantly better survival rate than those with poor response to steroids.

Rapid bone marrow response to induction therapy is also a favorable prognostic indicator. Previous Children's Cancer Group (CCG) studies showed that patients who had an M3 marrow (>25% blasts) on day 14 had a worse disease-free survival rate than children with an M1/M2 marrow ( $\leq$ 25% blast) on day 14 (13–15). Recently, CCG studies demonstrated that an early marrow response (M1/M2) on day 7 in patients at high-risk with ALL was associated with more favorable outcome (16). Intensification of therapy in patients at high-risk with poor day-7 response (M3) significantly improved their outcome (17). Steinherz et al. (18) attempted to determine the optimal time for assessment of the residual marrow lymphoblast fraction during remission induction. They demonstrated that the day-7 marrow findings had greater prognostic significance than day-14 marrow, and the day-14 marrow result provides additional prognostic information for those with an M3 day-7 marrow.

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Despite their prognostic usefulness, bone marrow aspiration samples during induction can be challenging to interpret because of hypocellularity, cell friability, and the difficulty of distinguishing regenerating lymphoblasts, hematogones, or mature lymphocytes from leukemic blasts that have been affected by chemotherapy. To account for variable marrow cellularity, Schultz et al. (19) derived an index of remaining blast cellularity by assessing the day-7 response in both marrow biopsy and marrow aspirate. It is uncertain whether the information gained from the bone marrow biopsy improved the prognostic significance of bone marrow aspirate.

Because an early response to therapy has been shown to be a significant prognostic factor in long-term survival, precise assessment of the marrow status is important, especially if subsequent therapy is guided by early marrow response. Multidimensional flow cytometry has been used in diagnosis of leukemia and in the detection of minimal residual disease (20–22). Multidimensional flow cytometry is more sensitive and specific at accurately distinguishing leukemic cells from the normal cell populations as compared with morphology, cytogenetics, or the two combined (23). We report a comparison of MDF with standard morphology to evaluate early marrow response during induction therapy for pediatric ALL.

## MATERIALS AND METHODS

### Patients

Between May 1997 and March 1999, untreated, newly diagnosed pediatric ALL were the criteria for patients eligible for enrollment on this study at Children's Hospital and Regional Medical Center, Seattle, Washington. Bone marrow samples were obtained after informed consent from parents/guardians in accordance with the Institutional Review Board. Bone marrow samples were obtained only when bone marrow aspiration was required by treatment protocol or standard practice. Bone marrow samples (1–2 mL) were aspirated in the conventional manner. The initial bone marrow aliquots were placed immediately in tubes containing edetic acid and used for morphologic evaluation. Subsequent aliquots were placed in vials with sodium heparin with or without RPMI medium (GIBCO-BRL, Rockville, MD, U.S.A.) and shipped within 24 to 48 hours to the MDF laboratory. A separate morphologic review was not performed on samples sent to the MDF laboratory. Patients were stratified to high-risk, standard risk, or infant groups based on their age and white blood cell count at presentation (24). High-risk was defined as white blood cell count  $>50,000/\mu\text{L}$  or age older than 10 years, standard risk was defined as white blood cell count  $\leq 50,000/\mu\text{L}$  and age older than 1 year and younger than 10 years, and the infant group was defined as age younger than 1 year. Patients were enrolled on current CCG-ALL protocols or treated according to standard institutional ALL therapy based on CCG therapy. All alterations to therapy were based on the proto-

col guidelines. Postinduction therapy was modified in response to early bone marrow response (as assessed by standard morphology) using these conditions:

1. High-risk and M3 on day-7 marrow
2. Standard risk and M3 on day-14 marrow
3. Standard risk and M2/M3 on day-28 marrow
4. High-risk and M3 on day-28 marrow

### Morphology

Bone marrow smears were prepared in the standard fashion (Wright-Giemsa) from the aspirated material; different tubes were used for MDF and morphologic review. The percent of lymphoblasts was determined by one of four pediatric pathologists who interpreted the smear during routine diagnostic evaluation. The pathologists were unaware of MDF results. Specimens were classified as  $<5\%$  (M1),  $5\%$  to  $25\%$  (M2), or  $>25\%$  (M3). For purposes of this study, a single pathologist, blinded to patient information including morphologic and MDF results, reevaluated available day-7 and day-14 marrows.

### Multidimensional Flow Cytometry

Monoclonal antibodies were obtained from these sources: fluorescein isothiocyanate: CD3, CD5, CD8, CD20, CD22, CD25, and human leukocyte antigen-DR (Becton Dickinson Immunocytometry Systems, San Jose, CA, U.S.A.); CD33 (DAKO, Carpinteria, CA, U.S.A.); terminal deoxynucleotidyl transferase (GenTRAK, Plymouth Meeting, PA, U.S.A.); phycoerythrin: CD4, CD7, CD11b, CD13, CD19, CD34, and CD56 (Becton Dickinson Immunocytometry Systems); CD10 (DAKO); CD1a (Pharmin-gen, San Diego, CA, U.S.A.); peridinin-chlorophyll-a (PerCP): CD45 (Becton Dickinson Immunocytometry Systems).

The staining procedure for labeling cells has been previously published (25). Briefly, a preselected panel of lymphoid antibody combinations was incubated at room temperature, in the dark, with  $100\ \mu\text{L}$  of well-mixed bone marrow, collected in sodium heparin with or without RPMI medium. After a 20-minute incubation, the erythrocytes were lysed with warmed ( $37^\circ\text{C}$ )  $\text{NH}_4\text{Cl}$  and the cell suspension was centrifuged at 1,300 rpm. The supernatant was discarded, leaving a cell pellet at the bottom of the tube. The cells were washed once with phosphate-buffered saline/2% fetal calf serum/0.1% sodium azide solution, and then the cell pellet was gently resuspended in 0.5 mL of 1% paraformaldehyde. Cytoplasmic staining required further preparation by incubating cells for 40 minutes, before fixation with paraformaldehyde, with 2.0 mL of a permeabilization solution, Ortho PermeaFix (Ortho Diagnostics, Raritan, NJ, U.S.A.). After permeabilization and washing once with phosphate-buffered saline/2% fetal calf serum/0.1% sodium azide solution, the cells were labeled with the cytoplasmic antibodies terminal deoxynucleotidyl transferase, myeloperoxidase, CD3, and CD22 (20). Acquisition of the data was performed on a Becton Dickinson Facscalibur using Cell-

quest software (Becton Dickinson Immunocytometry Systems) with a minimum of 10,000 events per sample. Multidimensional data analysis was performed using WinList software (Verity House, Topsham, ME, U.S.A.), as described by Wells et al. (26).

The leukemic blast population from the diagnostic marrow (day 0) was identified by forward and side light-scatter properties in combination with expression of the of the common leukocyte antigen (CD45). This gated population of blast cells was further analyzed for quantitative expression of various leukocyte antigens. This initial examination established the unique "fingerprint" for comparison with subsequent samples obtained at intervals during treatment. Cells from day-7, day-14, and day-28 marrow with matching immunophenotypic characteristics were considered leukemic blasts, and their percentage of total nucleated cell population was calculated. All patients had a distinctive phenotype that was used to follow-up the proportion of the leukemic cells.

### Clinical Assessment

Patients' risk groups were correlated with MDF and morphologic data. The pathologists and MDF staff were blinded to the other's interpretation of the bone marrow results. All treatment decisions were based on the morphologic evaluation of response as dictated by the treatment protocol. Treating physicians were blinded to MDF results. Because of the anticipated small sample size and brief follow-up, patient survival data were not analyzed in this study.

### Statistical Analysis

For the purpose of this study, discordant samples were defined as those that were classified as M1/M2 by one method and M3 by the other. This definition corresponds with the dichotomous distinction between rapid responders (M1/M2), who receive less intensive postinduction therapy, and slow responders (M3), who receive more intensive postinduction therapy. Assuming that the methods are equivalent, a concordance rate of 100% would ideally be expected. The hypothesis that the percent of discordant samples was significantly larger than 1% (chosen to represent a small rate of discordance) was tested using exact binomial probabilities and significance levels of 0.05. Ninety-five percent confidence intervals (CI) were also calculated using exact binomial probabilities.

## RESULTS

The characteristics of the 60 study patients are shown in Table 1. All samples were evaluated by morphology. Bone marrow specimens were obtained on days 0, 7, 14, and 28 from 60, 59, 33, and 57 patients, respectively, and all were evaluated by morphology. Bone marrow specimens were evaluable by both MDF and morphology on days 7, 14, and 28 in 46, 24, and 44 patients, respectively.

Twenty-six of the 46 (56%) evaluable patients had

**TABLE 1.** Patient characteristics

Characteristics	Number
Sex	
Male	32 (53%)
Female	28 (47%)
Age, years	
Mean (range)	6.3 (0.33–16.8)
WBC at diagnosis	
>50,000	14 (23%)
<50,000	46 (77%)
Risk Category	
High risk	23 (38%)
Standard risk	34 (57%)
Infant ALL	3 (5%)
Phenotype	
T cell	6 (10%)
Early Pre-B	17 (28%)
Common ALL	35 (58%)
Null	2 (3%)

M1/M2 marrow by both MDF and morphology on day 7 (Table 2). Twelve of 46 patients (21%) had M3 marrows on day 7 by both methods. However, 8 of 46 (17%; 95% CI: 8%, 31%) day-7 samples were discordant ( $P < 0.001$ ). In six patients, the marrow was classified as M3 by morphology, whereas MDF demonstrated an M1/M2 response. Two patients had an M1/M2 marrow by morphology and had M3 by MDF.

On day 14, 18 of the 24 (75%) evaluable patients had an M1/M2 marrow by both morphology and MDF (Table 2). One patient (4%) had M3 response on day 14 by both morphology and MDF. However, 5 of 24 (20.5%; 95% CI: 7%, 42%) of day-14 samples were discordant ( $P < 0.001$ ). Three of 24 patients' (12.5%) day-14 marrows were classified as M1/M2 by morphology and as M3 by MDF. Two other marrows (8%) were classified as M3 by morphology but as M1/M2 by MDF. Forty-three of 44 day-28 specimens were M1 by both methods. One patient had an M2 marrow (25% blasts) by morphology but an M1 marrow (3.8% blasts) by MDF.

Of the 13 patients with discordant MDF and morphology results, five had their chemotherapy regimen altered as a result of the morphologic evaluation of marrow response (Table 3). Two patients at high-risk with morphologically M3 day-7 marrows and two patients at standard risk with morphologically M3 day-14 marrows were assigned to more intensive chemotherapy regimens. One patient at stan-

**TABLE 2.** Flow cytometric and morphologic evaluation of day 7 and day 14 marrow

Morphology	Multidimensional flow			
	Day 7		Day 14	
	M1/M2 (%)	M3 (%)	M1/M2 (%)	M3 (%)
	26 (56)	2 (4)	18 (75)	3 (12.5)
	6 (13)	12 (26)	2 (8)	1 (4)
	$P < 0.001^*$		$P < 0.001^*$	

\* $P$ -value from binomial probability testing whether the percent of discordant samples is significantly greater than 1%.

**TABLE 3.** Discrepancies in flow cytometry vs. morphology results of day 7, 14, and 28 marrow

	Patient #	Risk group	Morphology (% blast)	MDF (% blast)	Postinduction chemotherapy change
Day 7 marrow	004	HR	30	0	
	006	SR	<25	41	
	013	SR	6	28	
	034	HR	>50	10	Intensified
	037	HR	50	13	Intensified
	038	SR	30%–50	23	
	047	SR	40	22	
Day 14 marrow	057	SR	75	14	
	018	SR	10	32	
	022	SR	3	44	
	032	SR	25%–30	0	Intensified
	035	SR	50	6	Intensified
Day 28 marrow	040	HR	20	61	
	018	SR	25	4	Intensified

HR, high risk; SR, standard risk.

standard risk who had an M2 marrow (25% blasts) on day 28 was assigned to more intensive chemotherapy regimen. All five of these patients had an M1/M2 marrow by MDF at the time of chemotherapy regimen alteration.

### Reevaluation of the Pathologic Specimens

We compared the result of the blinded reevaluation with the result of the original pathologic evaluation to determine the consistency of assessing leukemic blasts by morphology (Table 4). Of the 55 day-7 marrows that were reevaluated, six (11%; 95% CI: 4%, 22%) were discordant ( $P < 0.001$ ) from the original morphologic evaluation. Three marrows, called M3 on original evaluation, were reread as M1/M2. The other three that were read as M1/M2 initially were reread as M3. Two of 32 day-14 samples (6%; 95% CI: 1%, 21%) that were reevaluated were discordant ( $P = 0.04$ ). One was called M3 (25%–30%) on original evaluation and was read as M1 (2% blasts) on reevaluation. The other day-14 marrow that was originally read as M2 (20% blasts) was called M3 (40%) on reevaluation. Direct comparison of discordant samples are shown in Table 5.

### DISCUSSION

Early response to chemotherapy, as determined either by clearance of peripheral or marrow blasts, is a prognostic factor in pediatric ALL. Accurate identification of slow responders who are at higher-risk for relapse allows for intensification of postinduction chemotherapy and potential improvement of outcome. Morphologic evaluation of the response to chemotherapy relies on visual identification of immature cells in the marrow. However, differentiation of residual leukemic blasts from hematogones or mature lymphocytes may be challenging by morphology alone. In addition, day-7 and day-14 bone marrows are usually quite hypocellular, limiting the number of cells for evaluation. Because postinduction chemotherapy may be determined by the day-7 or day-14 marrow response, the distinction between M3 and M1/M2 marrow has significant clinical im-

plication. Therefore, appropriate allocation of patients to more or less intensive postinduction therapy requires accurate determination of bone marrow response.

To assess whether MDF would be a more accurate means of assessing early marrow response, we compared marrow response on days 7, 14, and 28 by MDF and morphology. Multidimensional flow cytometry has been used in evaluation of disease status in pediatric ALL (21) and appears to be an accurate and sensitive method to identify leukemic blasts. Multidimensional flow cytometry has not routinely been used to categorize early response, however. We demonstrate a significant discordance between morphologic and MDF evaluation of early bone marrow response during induction. Thirteen of 70 day-7 or day-14 bone marrow samples (19%) were discordant between the morphology and MDF results. Based on the morphologic evaluation of the marrow status, five patients received intensive postinduction chemotherapy. All five patients had classifications of M1/M2 by MDF. If MDF, and not morphology, was used to determine postinduction chemotherapy, these five patients would have continued on their standard treatment protocols. In addition, two patients at standard risk classified as having M1/M2 by morphology but M3 by MDF would have received intensified postinduction chemotherapy. In total, 7 of 46 patients (15%; 95% CI: 6%, 28%) evaluated by both MDF and morphology would have received different postinduction therapy if MDF was used to evaluate response rather than morphology.

**TABLE 4.** Morphologic reevaluation of day 7 and 14 marrow specimens

Original morphology	Reevaluation morphology			
	Day 7		Day 14	
	M1/M2	M3	M1/M2	M3
M1/M2	31	3	28	1
M3	3	18	1	2
	$*P < 0.001$		$*P < 0.04$	

\* $P$ -value from binomial probability testing whether the percent of discordant samples is significantly greater than 1%.

**TABLE 5.** Discordant samples between original and reevaluation morphology

	Patient #	Risk category	Original morphology, %	Reevaluation morphology, %	MDF evaluation, %
Day 7	4	HR	30	15	0
	10	SR	<5	27	NA
	13	SR	6	35	28
	32	SR	<15	56	0.20
	34	SR	>50	22	10
	49	HR	30	19	29
Day 14	32	SR	25%–30	2	0
	40	HR	20	40	61

NA, Not available.

We also show the subjective nature of the morphologic evaluation of bone marrow response during induction. Six of the 55 (11%) day-7 and 2 of 32 (6%) day-14 marrows reevaluated by a single pathologist were discordant from the original morphologic evaluation. Of the six discordant day-7 samples, two patients received intensive postinduction therapy based on the original evaluation. These patients would have continued on standard treatment based on the result of the reevaluation morphology. Also, one patient who received intensive postinduction therapy based on the original day-14 morphology would have remained on standard treatment based on the result of the reevaluation morphology.

Multidimensional flow cytometry may provide a more objective and reproducible assessment of the bone marrow than morphology in assessing response to induction therapy. Leukemia cells express aberrant phenotypes that can be used to identify them as neoplastic cells (27). Once the tumor is identified, it is possible to monitor its elimination from normal cells using the aberrant antigen expression as a tumor marker. Model studies have shown that abnormal cells can be distinguished and enumerated at frequencies less than 0.1% (28). An additional advantage of MDF over morphology is the ability to classify immature cells as normal or abnormal with a high predictive rate of relapse when abnormal cells are identified (26,29).

Our data demonstrate that the discordance between the morphology and MDF has random distribution and does not suggest any inherent bias for the observed difference. In no case did a patient demonstrate discordance in both the day-7 and day-14 sample, suggesting that discordance was not based on a unique morphology or immunophenotype that consistently “fooled” the pathologist or the MDF assessment. Moreover, because the sample for MDF was aspirated subsequent to the one sent for morphology, one might predict that morphology would be biased towards a higher percentage of blasts. However, in 5 of 13 discordant cases, morphology had a lower blast percentage than MDF. In addition, after morphologic reevaluation of the day-7 and day-14 marrows, four patients’ marrow status was changed from M3 to M1/M2, and the status of another four was changed from M1/M2 to M3. This suggests that the observed differences are because of the subjective nature of

the morphologic assessment of the induction marrow. Furthermore, evaluation of the MDF data from patients who were classified as having M1/M2 by MDF, but M3 by morphology, reveals that in the majority of patients (6/9) there were significant number of nonviable cells by MDF (low side-scatter and low CD 45 expression) (30). In additional two patients, there was evidence of regenerating normal B lymphoid cells (hematogones). The nonviable cells or hematogones may have contributed to the higher blast percentage by morphology. Because of the small sample size and limited follow-up, we are unable to correlate MDF and morphology with patient outcome. Correlation of clinical outcome by marrow status as determined by MDF versus morphology and exact reason for the discrepancy is part of the next CCG biology study.

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