Detection of Leukemic Lymphoblasts in CSF Is Instrument-Dependent

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Abstract

Staging and monitoring of pediatric acute lymphoblastic leukemia (ALL) includes examination of the cerebrospinal fluid (CSF). At our institution, we noted an increased incidence of low-level leukemic blasts in CSF samples from patients with ALL. This increase coincided with a conversion from the Shandon CytoSpin 4 (Thermo Fisher Scientific, Waltham, MA) to the Wescor Cytopro Rotor AC-060 (Wescor, Logan, UT). This study directly compared these 2 machines using patient samples and known concentrations of cultured leukemia cells. With patient samples, the Wescor Cytopro led to a 5- to 9-fold increase in the number of cells on a slide compared with the Shandon CytoSpin; furthermore, leukemic blasts were detected only with the Wescor Cytopro in 2 cases. Similar findings were observed using cultured leukemia cells. Thus, the detection of blasts in CSF is highly instrument-dependent. The newer, more sensitive cytocentrifuge machines identify blasts that were previously missed by older machines, but the clinical significance remains under investigation.

The 5-year survival rate of pediatric patients diagnosed with acute lymphoblastic leukemia (ALL) has increased from 54.1% in the 1975-1977 period to 85.1% in the 1999-2005 period[1] because of numerous treatment advances. Despite this progress, approximately 1 in 5 patients will have a recurrence of ALL, including some as isolated central nervous system (CNS) relapses. High cerebrospinal fluid (CSF) blast count at initial diagnosis (CNS 3, cell count ≥5 WBCs/μL with blasts present) is correlated with a poorer prognosis. However, the prognostic significance of a lower blast count in the CSF (CNS 2a, cell count <5 WBCs/μL, <10 RBCs/μL, and blasts identifiable on cytocentrifuged samples) is unclear, with varying implications reported among different studies.[2-4] In either case, initial successful therapy includes the clearance of blasts from the CSF (CNS 1).

In present-day Children's Oncology Group and international cooperative trials, the CSF cell count and cytocentrifuge data are obtained with each administration of intrathecal chemotherapy. CSF with CNS 3 findings represents ALL CNS relapse, and patients with these findings are eligible for ALL relapse trials with intensified systemic and focused CNS therapy. Some cooperative groups also define a subset of patients as having an "event" if there are 2 episodes of CNS 2 findings (ideally blasts are confirmed by immunophenotyping) during a 4-week interval, but patients with these findings are not currently eligible for relapse trials until they have a diagnosis of CNS 3.

At The Children's Hospital of Philadelphia, Philadelphia, PA, we noted an apparent increased incidence of CSF with CNS 2a findings during the last 3 years. This increase coincided with the introduction of a new cytocentrifuge machine in our pathology laboratory. In this report, we describe a causal relationship between the new cytocentrifuge machine and the

increased incidence of CNS 2a disease, indicating that the detection of blasts in the CSF is highly instrument-dependent.

Materials and Methods

Clinical Material

Selection criteria included cases with surveillance CSF showing cell counts of fewer than 5 WBCs/μL and fewer than 10 RBCs/μL with or without blasts and in which a sufficient amount of CSF was available for testing on both cytocentrifuge machines. Samples from the same CSF specimen tube obtained from 5 patients were compared using 1 mL for each cytocentrifuged slide. The study was performed in accordance with the policies of the institutional review board of The Children's Hospital of Philadelphia.

Cultured Cell Line

A human blastic cell line, K562, was obtained from the American Type Culture Collection and cultured in RPMI medium supplemented with 10% fetal calf serum. The cells were serially diluted in RPMI medium to final concentrations of 2.0, 1.0, 0.50, 0.25, and 0.10 cells/μL.

Cytocentrifuged Sample Preparation

For the study, a 1-mL sample and 2 drops of Ortho Bovine Albumin Solution (Ortho Clinical Diagnostics, Raritan, NJ) were centrifuged onto glass slides using the Wescor Cytopro Rotor AC-060 (Wescor, Logan, UT) or the Shandon CytoSpin 4 (Thermo Fisher Scientific, Waltham, MA) cytocentrifuge machines. Centrifuge settings for each machine were the same as those used with everyday patient samples: Shandon, 600 rpm for 10 minutes with low acceleration; Wescor, 1,000 rpm for 5 minutes with high acceleration. These settings were optimized (varying speed and duration of centrifugation) in our laboratory from validation studies performed when each machine was introduced. All slides from both cytocentrifuge machines were air dried and then stained with Wright-Giemsa using the Wescor Aerospray 7150 Hematology Slide Stainer. The cell count and differential were performed (by A.R.H. or M.H.) and verified (by J.K.C.).

Immunohistochemical Analysis

For immunohistochemical studies, 1 mL of patient CSF sample was centrifuged onto charged glass slides using the Wescor Cytopro. The air-dried cytocentrifuged slides were fixed in formalin, rinsed in deionized water, and then rinsed in tris(hydroxymethyl)aminomethane-buffered saline. The prepared slides were stained using antibodies against terminal deoxynucleotidyl transferase (TdT; dilution 1:60; 20 minutes; DAKO, Carpinteria, CA) using the Bond-maX automated immunohistochemistry system (Leica Microsystems, Buffalo Grove, IL). Antigen retrieval was performed using ER-2 retrieval buffer (Leica Microsystems), and the bound antibodies were detected using the bond polymer refine detection method (Leica Microsystems) following the manufacturer's protocol. The slides were then counterstained with hematoxylin.

Statistical Analysis

Comparison of results from the 2 machines was performed using the Student *t* test.

Results

In all 5 patient samples examined, the cytocentrifuged slide from the Wescor Cytopro showed a 5- to 9-fold increase in the number of cells retrieved compared with the equivalent slide from the Shandon CytoSpin 4 **Table 1**. The difference

Table 1
Comparison of CSF Results in Patient Samples Using Two Cytocentrifuge Machines

	Cell Count (/μL)			Differential (%)			
Case No./Machine	WBC	RBC	Total No. of Cells	Lymphocytes	Monocytes	Macrophages	Blasts
1	0	0					
Wescor			58	52	26	22	0
Shandon			15	47	27	26	0
2	0	0					
Wescor			100	35	11	51	3
Shandon			14	7	50	43	0
3	0	0					
Wescor			91	30	47	23	0
Shandon			10	30	50	20	0
4	0	2					
Wescor			69	22	49	25	4
Shandon			10	60	30	10	0
5	0	0					
Wescor			75	47	35	15	3
Shandon			15	40	27	27	7

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in total number of cells counted was statistically significant in our patient samples (P = .00056) and in the number of blasts counted (P = .044). In addition, 2 of 3 samples with detectable blasts were identified only on slides prepared on the Wescor Cytopro, not from slides prepared on the Shandon CytoSpin 4 (Table 1) **Image 1**. The presence of blasts was confirmed by immunohistochemical staining using antibodies against TdT for cases 2 and 5 (Image 1D). Apart from the blasts, the differential counts for other cell types were similar between the 2 cytocentrifuge machines (Table 1).

The increased cell recovery by the Wescor Cytopro compared with the Shandon CytoSpin 4 could result from different settings for the 2 machines. To explore this possibility, CSF samples from multiple patients were pooled, resulting in 10 mL of CSF with a cell count of 2 WBC/μL. One milliliter was centrifuged onto glass slides using the Shandon CytoSpin 4 at 3 settings: (1) 600 rpm for 10 minutes with low acceleration (current setting for this machine); (2) 1,000 rpm for 5 minutes with high acceleration (current setting for the Wescor Cytopro); and (3) 1,000 rpm for 10 minutes with high acceleration. The best cell recovery occurred with our current Shandon CytoSpin 4 setting (**Figure 1**).

The studies of the patient samples indicated that the Wescor Cytopro was much more efficient at centrifuging cells onto a glass slide compared with the Shandon CytoSpin 4. To confirm this finding and to quantify the efficiencies of cell recovery onto slides, we compared the 2 cytocentrifuge machines using known cell numbers and concentrations (100-2,000 cells and 0.1-2.0/μL, respectively). These cell concentrations were well below the concentration expected to yield 5 WBC/μL (CNS 3 status). We used a cultured ALL cell line, K562, permitting centrifugation in quintuplicate for each machine, a study that could not be performed using the limited volumes inherent in patient CSF samples. The disparity between the 2 machines is obvious when viewing the slides **Image 2**. The overall impression was confirmed by counting the total numbers of cells deposited on the slides under the various conditions. The mean and standard deviation for results from the known cell concentrations were calculated and are illustrated in **Figure 2**. As expected, the recovery rate of cells onto slides was lower than the starting input cells, but the Wescor Cytopro consistently yielded a higher average number of cells compared with the Shandon CytoSpin 4. For the Shandon CytoSpin 4, the recovery rate was 1.6% with 100 cells and 8.1% with 2,000 cells. In contrast, the recovery rate for the Wescor Cytopro was higher, 9.6% with 100 cells and 32% with 2,000 cells.

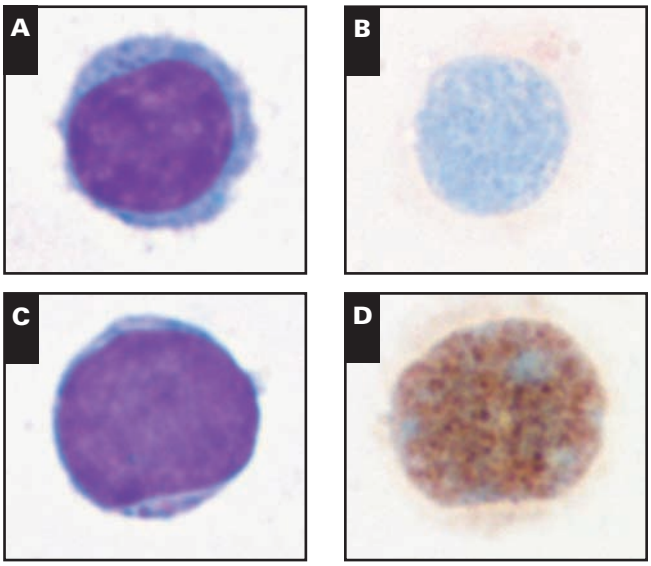

Image 1 A mature lymphocyte (**A** and **B**) and lymphoblast (**C** and **D**) seen in cytocentrifuged patient samples using the Wescor Cytopro (**A** and **C**, Wright-Giemsa stain, ×1,000; **B** and **D**, immunohistochemical analysis for terminal deoxynucleotidyl transferase, ×1,000).

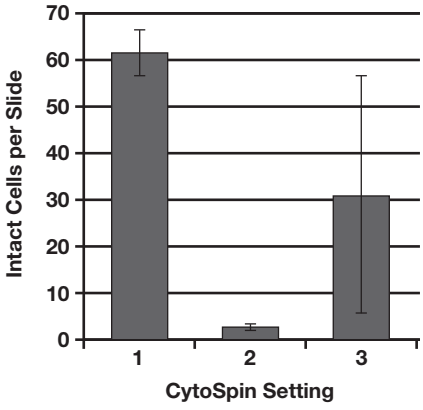

Figure 1 The number of cells counted per slide (y-axis) plotted against 1 mL of pooled cerebrospinal fluid (2 WBC/μL) centrifuged at 3 settings (1, 600 rpm for 10 min; 2, 1,000 rpm for 5 min; 3, 1,000 rpm for 10 min) on the Shandon CytoSpin 4 (x-axis). Each setting was done in duplicate.

Discussion

Our study demonstrates that the number of cells detected on a cytocentrifuged sample with a given volume of CSF is highly dependent on the type of cytocentrifuge machine, with higher cell numbers seen with the Wescor Cytopro compared with the Shandon CytoSpin 4. The higher number of total cells recovered could explain why 2 CSF samples had detectable blasts with the Wescor Cytopro but not with the Shandon CytoSpin. The sample for case 2 (Table 1) had 100 total cells, including 3 blasts, deposited on the slide using the Wescor Cytopro, indicating approximately 1 blast for every 33 cells. The same sample had only 14 cells deposited on the slide using the Shandon CytoSpin, so there is a risk that a blast

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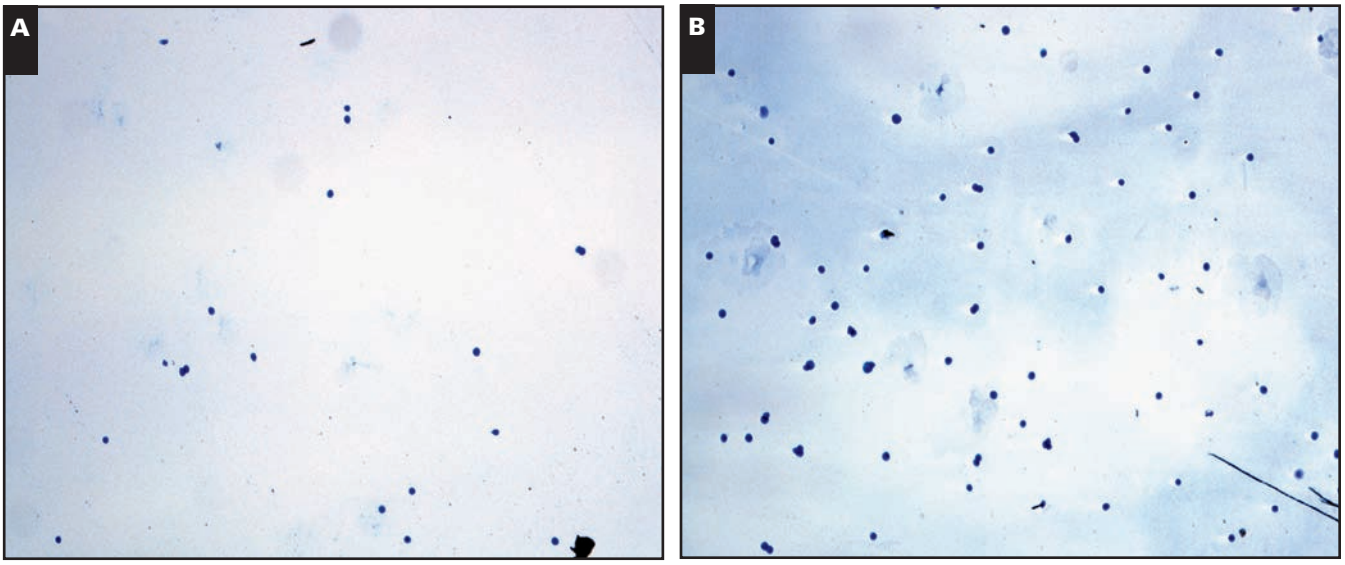

Image 2 Cytocentrifuged samples using the Shandon CytoSpin 4 (**A**) and the Wescor Cytopro (**B**) with 2,000 K562 cells in 1 mL (Wright-Giemsa stain, ×40).

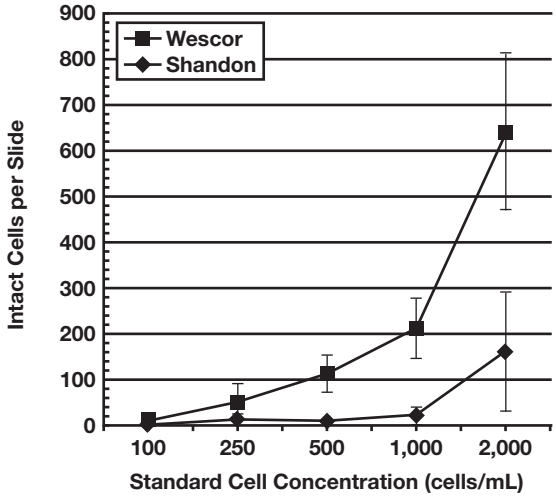

Figure 2 The mean number of cells counted per slide (y-axis) plotted against input number of K562 cells in 1 mL of RPMI (x-axis) for the Wescor Cytopro Rotor AC-060 vs the Shandon CytoSpin 4. Each sample was done in quintuplicate. Error bars indicate the standard deviation.

would not be detected. The increased efficiency of depositing cells onto slides correlates with the increased number of cases of CNS 2a disease seen in our patients that coincided with the switch to the Wescor Cytopro.

In 2 of 3 samples with blasts, the blasts were confirmed by TdT immunohistochemical staining. Case 4, with positive blasts noted in morphologic studies, did not have detectible TdT+ cells, suggesting 3 possible explanations: (1) Because a second cytocentrifuge slide was used for the TdT stain, the blasts may not have been represented on that slide. (2) Our immunohistochemical assay for TdT performed on the air-dried cytocentrifuge slides was not fully optimized and is not as sensitive as with paraffin-fixed sections, preventing the detection of dim TdT+ cells. (3) Some cells with blast-like morphologic features were not true lymphoblasts. While we favor the first explanation, we cannot exclude the other 2 possibilities.

While the exact explanation for the increased efficiency of the Wescor Cytopro machine is not clear, we can exclude higher centrifugation force with the Wescor (1,000 rpm) compared with the Shandon (600 rpm); the radii of the rotors for both machines (~100 mm) are similar. Increasing the speed of the Shandon CytoSpin to 1,000 rpm for 5 or 10 minutes did not increase the number of cells deposited and, instead, led to a decreased number of intact cells and increased cell debris.

The more likely explanation for increased efficiency is that the Wescor Cytopro funnel has a smaller dead space compared with that for the Shandon CytoSpin, leading to decreased loss of cells. The potential causes of a difference in dead space include shape of the cytocentrifuge funnel **Image 3**, material composition of the funnel, and the absorbent Cytopad that removes the excess fluid of the sample. The Wescor Web site states that the "compressed flow control ring in the Cytopad yields the highest cell recovery in the industry" (http://www.wescor.com/biomedical/slidestainers/cytopro_rotor_page.html).

During this study, we also identified variability in the volume of CSF centrifuged at our institution, even before the use of the Wescor machine. Depending on the technician, cell count, and site (hospital vs satellite laboratory), we were using

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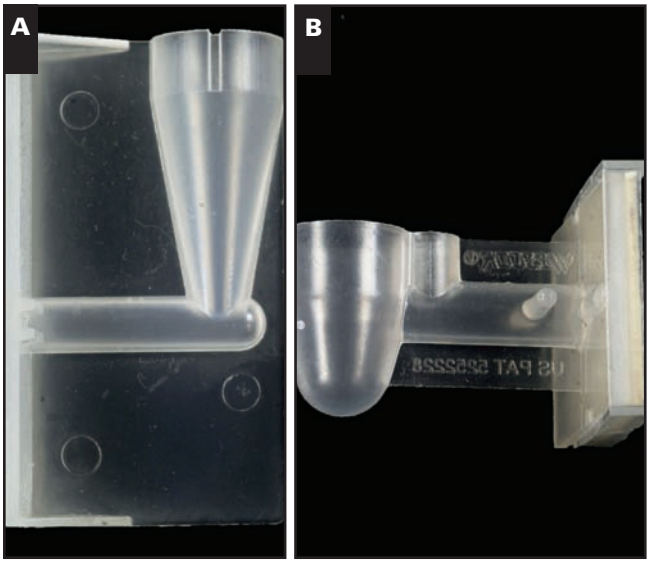

Image 3 Cytocentrifuge funnels for the Shandon CytoSpin 4 (**A**) and Wescor Cytopro (**B**) differ in shape and size.

between 0.1 and 1.5 mL of cytocentrifuged fluid to count a differential, a process we have now standardized institutionally to 1 mL. While we used a pipette to accurately measure 1 mL for this study, we continue to use drop estimation (assuming 1 drop = 50 µL) in the clinical setting for workflow reasons. No national or cooperative group guidelines for the volume of CSF used for cytocentrifuging exist; the College of American Pathologists also has no stated guidelines. Because the difference in CNS status can be clinically significant and drive protocol therapy, standardization may be necessary.

The variations in the cytocentrifuge machines, rotor settings, and laboratory practice could all contribute to variable numbers of blasts in the CSF samples with low WBC counts, potentially explaining the differing clinical significance of CNS 2 at initial ALL diagnosis in previous studies.[2-4] However, only 1 of these 3 studies provides any details about the method or equipment used in cytocentrifuge sample preparation. Additional technical advances will increase the efficiency of cell recovery and further complicate the clinical significance of CNS 2 at diagnosis. Future studies regarding the significance of blasts in the CSF of patients with ALL should clearly state the equipment and methods used in cytocentrifuge sample preparation so that the results may be correlated.

In current Children's Oncology Group therapeutic trials for children with lower risk ALL, CNS 2 status at diagnosis is an exclusion criterion for assignment to reduced intensity regimens. During postremission therapy, persistent CNS 2 status may be considered an event that removes children from protocol therapy. Changes in our machine and method have increased the incidence of CNS 2 disease at our institution with evidence that some of the cases would not have been detected with the older method. We are currently collecting additional case and individual patient follow-up data to determine the clinical significance of these findings.

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