Possible Automatic Cell Classification of Bone Marrow Aspirate Using the CELL-DYN 4000[®] Automatic Blood Cell Analyzer

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In clinical hematology, the demand for bone marrow aspiration testing is increasing. However, conventional automatic blood cell analyzers cannot completely analyze erythroblasts, and evaluation has mainly been performed by visual examination (the microscopic method). Using the CELL-DYN 4000[®] automatic blood cell analyzer (CD4000) (Abbott Laboratories, North Chicago, IL), specific recognition and classification of erythroblasts by DNA staining is possible. In the present study, using bone marrow blood collected from normal subjects and patients with hematological malignancy, we classified cells by the microscopic method and with the CD4000, and compared the results. Good

correlations were found for total nucleated cell count (TNCC), neutrophils, lymphocytes, erythroblasts, and the myeloid series to erythroid series (M/E) ratio. It is possible to detect blasts that emerge in patients with hematological malignancy using the blast flag system installed on the CD4000. Since all of the items can be analyzed in about 80 sec with the CD4000, cells in bone marrow aspirates can be classified faster with this apparatus than by the microscopic method. Therefore, analysis of bone marrow aspirates with this apparatus appears to be very useful not only for laboratory testing but also for clinical screening. *J. Clin. Lab. Anal.* 16:86–90, 2002. © 2002 Wiley-Liss, Inc.

Key words: automatic blood cell analyzer; blast; erythroblast; flow cytometry; leukemia

INTRODUCTION

Historically, the classification of bone marrow cells has been performed by visual methods using a light microscope. Analysis with automatic blood cell analyzers was impossible (1–3) because the bone marrow aspirate contains fat particles and cells with nuclei of various shapes and sizes. Even with the use of an apparatus that could classify leukocytes into five groups, clear differentiation of erythroblasts from lymphocytes was impossible because the sizes and shapes of the nuclei in these two cell populations are very similar. With the CELL-DYN 4000[®] (CD4000) (Abbott Laboratories, North Chicago, IL), which combines five-dimensional laser photometry with a multi-angle photo-scattering separation procedure together with DNA staining, classification of erythroblasts is made possible by destroying the membranes of erythroblasts in the hemolytic process and staining the DNA of the erythroblast nuclei. The erythrocytes are also classified into five groups (4,5). In the present study, we analyzed bone marrow aspirates obtained from normal subjects and patients with hematological malignancy using the CD4000, and examined the possible clinical application of this apparatus.

MATERIALS AND METHODS

Materials

As normal samples, this study included 93 specimens that had been collected for bone marrow aspiration tests in Osaka City University Medical School from April 1997 through October 2000. The specimens exhibited no dysplasia in myeloid, lymphocyte, or erythroblast series, and contained no abnormal cells on light-microscopic examination. The abnormal specimens examined were 106 samples collected for bone marrow aspiration tests from patients diagnosed as having acute leukemia, chronic myeloid leukemia, or myelodysplastic syndrome (MDS).

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Methods

CD4000

After patient consent was obtained, 0.5 ml of bone marrow aspirate was used for analysis with the CD4000. Each specimen was anticoagulated with dipotassium ethylene diamine tetraacetic acid (EDTA-2K) and diluted twofold with 0.5 ml of phosphate-buffered saline (PBS). Analysis with the CD4000 was performed in the resistant red blood cell (RBC) mode within 5 hr after sample collection (6). Items examined in the present study were mature neutrophils (stab and segmented), immature neutrophils, eosinophils, basophils, lymphocytes, monocytes, erythroblasts, blasts, and myeloid/erythroid ratio (M/E ratio), which are determinable with the CD4000 using original technologies that are described in detail elsewhere (7,8). The total nucleated cell count (TNCC) in bone marrow aspiration specimens is calculated with the CD4000 from the sum of the leukocyte and erythroblast counts. The numbers of blasts are primarily estimated using 90° and 0° scattered light. The CD4000 generates a flag for the presence of blasts. A blast flag is derived by a proprietary algorithm, depending on the number of monocytes (>20%), the relative position of the cell in the lymphocyte/monocyte algorithm, and the level of fluorescence in the WBC measurement channel. Furthermore, a blast flag confidence fraction (CF) is generated which runs from 0.50 to 0.99 and indicates the level of probability derived from discriminant analysis (9,10).

Microscopic method

Bone marrow aspirate was smeared on a glass slide and subjected to May-Grünwald-Giemsa staining, and 500 cells were examined by light microscopy and classified. For TNCC, a 1:50 dilution of the marrow with Türk's diluting fluid was performed in a Melangeur blood diluting pipette, and TNCs were manually counted using a Bürker Türk hemocytometer.

Statistical analysis

Values are expressed as the mean $\pm$ standard deviation (SD). In statistical assessments, the *t*-test and Bearmann method were used with a significance level of 0.05 or less.

RESULTS

Comparison Between the CD4000 and the Microscopic Method in Classification of Cells in Bone Marrow Aspirates

Comparison using normal specimens

The CD4000 and the microscopic method were compared for classification of cells in normal specimens. The percentages, correlations, coefficients, and *P* values are shown in Table 1. The TNCC, total neutrophils, mature neutrophils, lymphocytes, erythroblasts, and M/E ratio exhibited good correlations, while the number of mature neutrophils calculated with the CD4000 was higher than with the microscopic method. The number of immature neutrophils calculated with the CD4000 was lower than that with the microscopic method.

Comparison using abnormal specimens. The CD4000 and the microscopic method were compared for classification of cells in abnormal specimens. Correlation coefficients (*r*'s) for the numbers of TNCC, total neutrophils, lymphocytes, and erythroblasts were 0.91, 0.85, 0.93, and 0.66, respectively, showing good correlations (Table 2). However, no correlation was found in the analysis of immature neutrophils, eosinophils, basophils, monocytes, blasts, or M/E ratio. In four specimens, blasts accounted for more than 60% of cells by the microscopic method, whereas some of the blasts were counted as erythroblasts, atypical lymphocytes, mature neutrophils, monocytes, or atypical lymphocytes by the CD4000.

Comparison Between CD4000 and the Microscopic Method in Blast Detection

Examination of blast percentage

The percentages of blasts in 93 specimens collected by bone marrow aspiration from normal subjects, and 106 specimens from patients with hematological malignancy calculated with the CD4000 were compared with those obtained by the microscopic method. The percentages of blasts in normal subjects obtained with the CD4000 and by the mi-

TABLE 1. Comparison of subpopulation percentages obtained with the resist RBC mode of CD4000 and by the microscopic method (Means $\pm$ SD)

	CD4000 resist mode	Microscopic method	<i>r</i>	<i>P</i> value
Total nucleated cell count ($\times 10^9/l$)	99.9 $\pm$ 87.5	110.2 $\pm$ 97.5	0.93	<0.0001
Mature neutrophils (%)	39.2 $\pm$ 10.2	29.6 $\pm$ 8.2	0.62	<0.0001
Immature neutrophils (%)	6.7 $\pm$ 3.2	21.5 $\pm$ 6.1	0.42	<0.0001
Total neutrophils (%)	45.9 $\pm$ 11.6	50.0 $\pm$ 11.2	0.70	<0.0001
Eosinophils (%)	2.7 $\pm$ 5.2	2.5 $\pm$ 1.5	0.24	0.02
Basophils (%)	0.10 $\pm$ 0.13	0.30 $\pm$ 0.31	0.018	0.87
Lymphocytes (%)	13.4 $\pm$ 6.3	14.5 $\pm$ 6.6	0.83	<0.0001
Monocytes (%)	8.1 $\pm$ 3.5	4.0 $\pm$ 1.5	0.04	0.7
Erythroblasts (%)	24.6 $\pm$ 6.5	26.86 $\pm$ 9.6	0.56	<0.0001
Blasts (%)	2.1 $\pm$ 2.8	0.64 $\pm$ 0.32	0.12	0.31
M/E ratio	2.2 $\pm$ 1.2	2.4 $\pm$ 1.4	0.74	<0.0001

TABLE 2. Comparison of subpopulation percentages obtained with the resist RBC mode of CD4000 and by the microscopic method, in abnormal specimens (Means $\pm$ SD)

	CD4000 resist mode	Microscopic method	<i>r</i>	<i>P</i> value
Total nucleated cell count ($\times 10^9/1$)	124.2 $\pm$ 162.0	136.2 $\pm$ 193.9	0.91	<0.0001
Total neutrophils (%)	45.2 $\pm$ 18.0	47.1 $\pm$ 19.0	0.85	<0.0001
Lymphocytes (%)	11.5 $\pm$ 10.4	11.8 $\pm$ 10.9	0.93	<0.0001
Erythroblasts (%)	22.5 $\pm$ 12.4	25.9 $\pm$ 16.4	0.68	<0.0001
Blasts (%)	5.6 $\pm$ 5.8	4.8 $\pm$ 11.8	0.16	0.1

croscopic method were 2.1% $\pm$ 2.8% and 0.64% $\pm$ 0.32%, respectively, with $r = 0.12$ ($P = 0.31$). The upper limits of the percentage of blasts with the CD4000 and the microscopic method were 7.7% and 1.3% (Table 1), respectively, in normal specimens, provided the upper limit of the normal range was set as the mean + 2 $\times$ SD. In abnormal specimens, the percentages of blasts obtained with the CD4000 and by microscopy were 5.6% $\pm$ 5.8% and 4.8% $\pm$ 11.8%, respectively (Table 2). Using 1.3% (which is the upper limit of the percentage of blasts determined by microscopy in normal specimens) as the standard, the sensitivity and specificity were determined using abnormal specimens. At a percentage of blasts of 3% with the CD4000, the sensitivity was 72.7% and the specificity was 46.8% (Table 3, Fig. 1). Therefore, when the blast rate was 3% or more with the CD4000, the likelihood of observation of abnormal cells by microscopy is high.

Examination by blast flag

The possibility of detecting blasts by the blast flag system installed on the CD4000 was examined. Among the 192 cases examined in the present study, which included both normal and abnormal samples, a blast flag was obtained in 117 (60.9%) (Table 3). The blast flag was obtained in 38 normal cases (43.2%) and 79 abnormal cases (76.0%). When limited to the 44 specimens with blast percentages above 1.3% by microscopic examination, a blast flag was obtained in 37 cases (84.1%). When the normal upper limit of the percentage of blasts of 1.3% is set as the standard, if a blast flag CF of 0.65 is used as a cut-off value, differentiation is possible with a sensitivity of 75.0% and a specificity of 62.8% (Table 3).

TABLE 3. Contingency table analysis: detection of abnormal specimens by the CD4000

Cut off level of CD4000	Microscopic analysis	
	Blast < 0.3%	Blast $\geq$ 1.3%
Blast < 3% ^a	29	12
Blast $\geq$ 3% ^a	33	32
CF (negative) ^b	68	7
CF (positive) ^b	80	37
CF < 0.65 ^c	93	11
CF $\geq$ 0.65 ^c	55	33

^aSensitivity = 72.7%; specificity = 46.8%.

^bSensitivity = 84.1%; specificity = 45.9%.

^cSensitivity = 75.0%; specificity = 62.8%.

DISCUSSION

Clinical demand for bone marrow aspiration testing to diagnose various types of hematological disease or monitor the effect of chemotherapy on bone marrow in malignant diseases is increasing. However, evaluation of bone marrow aspiration specimens is mainly performed by visual examination using light microscopes, and time is required from staining to classification. Since existing automatic blood cell analyzers cannot recognize erythroblasts, their classification of bone marrow cells is not accurate. If automatic analysis of bone marrow aspiration specimens were possible, a significant shortening of analytical time would be expected. The CD4000, developed by Abbott Laboratories, permits classification of leukocytes into five groups using flow cytometry and classification of erythroid series cells into reticulocytes (11), immature reticulocytes, and nucleated RBCs (NRBCs) (5). Therefore, in the present study, we examined automatic classification with the CD4000 using 199 bone marrow aspirates.

With the CD4000, erythroblasts are lysed and their nuclei are stained with PI and counted. For analysis of NRBCs, two modes with different hemolytic times, that is, normal mode with a hemolytic time of 11.5 sec and resist RBC mode with a longer time (34 sec) of exposure to the hemolytic agent are installed on the CD4000. The CD4000 with normal mode and

Fig. 1. Relationship between the percentages of blasts obtained with the CD4000 and by the microscopic method. Samples were obtained from abnormal bone marrows. The horizontal line represents the cut-off level of CD4000, blast 3%.

microscopy yielded erythroblast percentages that were well correlated, but the percentage with the CD4000 was lower than that obtained by microscopy (7). This appeared to be due to the fact that erythroblasts resistant to the hemolytic agent in a certain stage of differentiation are present in bone marrow, and this type of cell is not counted as an erythroblast. Using the resist mode, we analyzed bone marrow aspirates and found that the erythroblast percentage obtained with the CD4000 was close to that obtained by microscopy (6). Therefore, in the present analysis of bone marrow aspirates, the resist RBC mode was used. Paterakis et al. (12) reported a good correlation ($r = 0.82$) between the percentage of erythroblasts obtained with the CD4000 and that obtained by the microscopic method.

In normal specimens, a good correlation was found between the percentages obtained with the CD4000 and those obtained by microscopy for mature neutrophils, lymphocytes, erythroblasts, and the M/E ratio. However, no significant correlation was found in the percentage of eosinophils, basophils, monocytes, or blasts. This apparently was due to their low percentages relative to TNCC. Sakamoto et al. (7) reported similar observations from 48 normal samples. Jones et al. (13) reported that the percentage of basophils was as low as 0.63% with the CD4000 and 1.04% with microscopy, and that the correlation between them was poor ($r = 0.12$).

For both mature and immature neutrophils, good correlations were observed between the results obtained with the CD4000 and those by microscopy (mature neutrophils: $r = 0.62$; immature neutrophils: $r = 0.42$), but the actual values obtained were clearly different (mature neutrophils: $39.2 \pm 10.2\%$ with the CD4000, and $28.6 \pm 8.2\%$ by microscopy; immature neutrophils: $6.7 \pm 3.2\%$ with the CD4000, and $21.5 \pm 6.1\%$ by microscopy). In bone marrow aspirate, the percentages of myelocytes and metamyelocytes are high. In many cases, separation of metamyelocytes from stab neutrophils by microscopy is performed based on the examiner's subjective judgment—for example, in determining the degree of nucleus nick. Therefore, classification of some metamyelocytes into stab neutrophils with the CD4000 seems to be a reason why percentages of mature neutrophils obtained with the CD4000 is different from those by microscopy. Actually, the percentage of total neutrophils (i.e., mature neutrophils plus immature neutrophils) obtained with the CD4000 was the same as that obtained by microscopy ($45.9 \pm 11.6\%$ with the CD4000; $50.0 \pm 11.2\%$ by microscopy), and a significant correlation was also observed ($r = 0.70$).

Detection of blasts in peripheral blood with the CD4000 has been examined in previous studies. Glazier et al. (14) mixed cells of a leukemia cell line with normal peripheral blood, and showed that detection of blasts with the CD4000 was possible, with a good correlation. Hoedemakers et al. (10) reported that blasts in peripheral blood could be detected at a probability of 97.4% using two parameters—the blast flag and the number of leukocytes—and that the time required

for microscopic confirmation could be saved. However, blasts may take various forms depending on the disease involved, and the accuracy of differentiating them from normal cells by the light-scattering data alone appears to be limited. When a comparison was made using specimens containing many blasts, some acute lymphocytic leukemia cells were included in the erythroblast fraction and the atypical lymphocyte fraction, some acute myelogenous leukemia cells were included in the monocyte and atypical lymphocyte fraction, and blasts at the time of conversion of chronic myelogenous leukemia cells to the acute type were included in the mature neutrophil fraction. The presence of acute lymphocytic leukemia cells in the erythroblast fraction appears to indicate that blast membranes were lysed by the hemolytic agent for erythrocytes and stained with PI. For this type of blast, identification with the CD4000 is difficult. Therefore, it is difficult to analyze with precision all of the various types of leukemia cells with automatic analyzers. However, automatic analysis of bone marrow blood as a screening test will save time for workers and be a great advantage. D'Onofrio et al. (15) reported that clusters of different types of leukemic blasts can be identified on the scatterplot that the CD4000 prints out. In the present study, we examined to what extent blasts could be screened with an automatic blood cell analyzer.

Since myeloblasts are present in the normal bone marrow, the standard value for blast percentage is necessarily different from that for peripheral blood. Blast percentages in normal specimens obtained with the CD4000 and by microscopy were $2.1 \pm 2.8\%$ and $0.64 \pm 0.32\%$, respectively, with $r = 0.12$, which was not significant ($P = 0.31$). The upper limit of blast percentage in normal specimens obtained with the CD4000 and by microscopy were 7.7% and 1.3%, respectively.

The possibility of using the CD4000 for bone marrow blood analysis as a screening test for hematological malignancy was investigated. In a previous study of blasts in the bone marrow, D'Onofrio et al. (8) reported that blast flag was positive in all of 20 samples with more than 5% blasts, and blast flag was negative in 45 of 64 samples with less than 5% bone marrow blasts. We examined whether much less blasts can be detected with the CD4000. The normal range of blast percentages obtained by microscopy was $0.64\% \pm 0.32\%$. Using the upper normal range of blast of 1.3% as the standard, when the cut-off value of blast rate with the CD4000 is set to 3%, a good sensitivity of 72.7% and a specificity of 46.8% were obtained. Using the blast flag CF function, with the upper limit of normal blast percentage of 1.3% set as the standard, and a blast flag CF of 0.65 used as a cut-off value, differentiation at a sensitivity of 75.0% and a specificity of 62.8% was possible. Regarding detection of blasts in peripheral blood using the blast flag CF function, when the cut-off value of blast CF is set to 0.75, it was reported that it was possible that blasts could be detected with the CD4000 at a sensitivity of 97.4% and a specificity of 56.5% (10).

Therefore, in screening bone marrow aspirates with the

CD4000 when blasts account for 3% or more of cells, or the blast flag CF is above 0.65, it is highly possible that blasts exist more than 1.3% by microscopy.

The CD4000 can complete the measurement of all items in about 80 sec, and classification of cells in bone marrow aspirates is performed faster than by microscopy. Therefore, screening of bone marrow aspirates with this apparatus appears to be very useful for clinical testing and screening.

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