Laboratory Hematology 1:128–134
© 1995 International Society for Laboratory Hematology

Computerized analysis of cells from patients with acute myelogenous leukemia prepared by density gradient centrifugation or erythrocyte lysis and measured by flow cytometry

H.-G. HÖFFKES,[1] G. SCHMIDTKE,[1] U. SCHMÜCKER,[1] G. BRITTINGER,[1] G. VALET[2]

[1] *Division of Hematology, Department of Medicine, University of Essen;*
[2] *Max-Planck-Institute, Division of Cellbiochemistry, Munich/Martinsried, Germany*

SUMMARY

We obtained bone marrow and peripheral blood samples from six patients with acute myelogenous leukemia and investigated the effects of density gradient centrifugation or erythrocyte lysis on multiparametric flow cytometry analysis results of leukemic leukocytes. Three-color immunophenotypes provide information on lymphocytes, monocytes, and granulocytes by the forward/sideward scatter (FSC/SSC) histogram and by quadrant evaluation of the fluorescein thiocyanate (FITC)/phycoerythrin (PE), PE/peridin chlorophyll A protein (PerCP) or PE cyanine 5 (PECy5), and FITC/PerCP or PECy5 histograms. Typically, the percent lymphocyte frequency numbers in 12 (3×4) quadrants or, in the case of absolute counts, 24 (2×12) are extracted. This information is not exhaustive because fluorescence intensities, fluorescence ratios, relative fluorescence densities on the cell membrane, and monocyte and granulocyte information are not evaluated. Analysis of data obtained by the three-color fluorescence measurements was performed by using the newly developed CLASSIF1 program for automated data classification. The parameters for the CLASSIF1 program combined the percent quadrant analysis with all the above additional parameters. In general, antibody density on the surface of leukocytes prepared by density gradient centrifugation was higher than by erythrocyte lysis. Density gradient preparation may lead to better staining, particularly for weak expression of antigens on the surface of myelogenous blasts. Antigenic changes, rather than cell depletion after density gradient centrifugation, were the relevant parameters to distinguish between density gradient centrifugation and erythrocyte lysis preparations. Correct single-case recognition for individual samples was 100% for peripheral blood and 83.5% for bone marrow samples, irrespective of preparation by density gradient centrifugation or erythrocyte lysis. Lab. Hematol. 1:128–134, 1995

KEY WORDS: Flow cytometry · Density gradient centrifugation · Erythrocyte lysis · Acute myelogenous leukemia · CLASSIF1-program

Address correspondence and reprint requests to Dr. Höffkes at the University of Essen, Division of Hematology, Department of Medicine, Hufelandstraße 55, 45122 Essen, Germany.
Received 30 June 1995; accepted 5 October 1995

INTRODUCTION

Immunophenotyping is a standard tool for classifying acute leukemias, and flow cytometry has become the preferred method for lineage assessment and characterization of maturation [1,2]. One of the most important applications of immunophenotyping is the differentiation of morphologically and cytochemically unclassified leukemias. Interestingly, little is known about the influence cell preparation by density gradient centrifugation or erythrocyte lysis may have on the antigen expression of blasts from patients with acute myelogenous leukemia [3,4]. In the past, most results have been obtained from cells prepared by density gradient centrifugation. Recently, erythrocyte lysis by appropriate red blood cell–lysing reagents has become a simple method for routine analysis of whole blood. Compared with density gradient centrifugation, erythrocyte lysis has the advantage of a substantially higher recovery of leukocytes without selective loss of lymphocytes [5,6].

In the present paper, peripheral blood and bone marrow were used to investigate the effects of density gradient centrifugation and erythrocyte lysis on three-color fluorescence cytometry analysis of leukemic cells from patients with *de novo* acute myelogenous leukemia using the CLASSIF1 data classification. The data obtained indicate that the preparation of leukocytes by density gradient centrifugation results in an increase of mean antigen fluorescence intensity compared with the preparation by erythrocyte lysis.

PATIENTS, MATERIALS, AND METHODS

Heparinized bone marrow (BM) and peripheral blood leukocytes (PBL) were collected from six leukemic patients with acute myelogenous leukemia as part of the clinical evaluation before

administration of chemotherapeutic agents. Each of the specimens was prepared by density gradient centrifugation (gradient bone marrow [GBM], n=6, and gradient blood leukocytes [GBL], n=6) and by erythrocyte lysis (BM, n=6, and PBL, n=6) resulting in a total of 24 samples processed. Acute myelogenous leukemia was diagnosed by standard criteria according to the French-American-British (FAB) classification [7].

Cell Preparation

All samples obtained were immediately transferred to the laboratory and processed within 2 hours after arrival. The preparation by density gradient centrifugation and by erythrocyte lysis was performed by the same person. Data were analyzed using both the Lysis II and the CLASSIF1 programs.

Preparation of Leukocytes by Ficoll-Hypaque Density Gradient Centrifugation

Leukemic cells were enriched by Ficoll-Hypaque (FH) density gradient centrifugation [8]. Heparinized samples of bone marrow and peripheral blood were diluted with phosphate-buffered saline (PBS) (Dulbecco's, without Ca^{++} or Mg^{++}, without sodium bicarbonate; Gibco BRL, Eggenstein, Germany) in a ratio of 1:2 at room temperature. Twenty milliliters of the diluted blood sample was placed into 50-mL centrifuge tubes (Falcon tube Blue Max; Becton Dickinson, San Jose, CA), and 20 mL of FH (Ficoll-Hypaque Pharmacia; Biotechnology International AB, Uppsala, Sweden) were underlayered. After the tip of the pipette containing the Ficoll had been positioned at the bottom of the tube, the high-density Ficoll was released, flowing to the bottom of the tube by gravity. When the level of Ficoll in the pipette reached the top of the blood layer, the pipette was slowly raised until the tip was at the interface. The technician placed a finger at the pipette top and slowly withdrew the pipette from the tube. The tube was then centrifuged at approximately 800*g* for 25 minutes at room temperature. Using a Pasteur pipette, all mononuclear cells at the interface were removed, collected, and resuspended in PBS and centrifuged again at 800*g* for 10 minutes at room temperature. The pellet was removed by a Pasteur pipette, resuspended in PBS, and washed twice by centrifugation at 700*g* and 500*g* for 10 minutes each with PBS. Following resuspension, the cells were counted (Coulter Counter STKS; Coulter Electronics GmbH, Krefeld, Germany) and adjusted to a concentration of 5×10^3/μL in PBS. The cell viability was examined by trypan blue staining (Gibco BRL, Eggenstein, Germany). Monoclonal antibodies (20 L) were added to 100 L of the adjusted suspension, which was then incubated for 30 minutes at 4°C. The suspension was vortexed every 5 minutes. The cells were washed with 2 mL PBS containing 0.5% bovine serum albumin (BSA), 0.1% NaN_3, and centrifuged at 400*g* for 5 minutes. The pellet was washed by PBS, centrifuged at 400*g* for 5 minutes, resuspended in 400 L PBS/BSA/NaN_3, and analyzed within 30 minutes.

Staining of Unseparated Whole Blood Followed by Erythrocyte Lysis

Heparinized samples of bone marrow and peripheral blood were prepared for flow cytometric analysis using erythrocyte lysis [8]. The cells were first counted (Coulter Counter STKS) and adjusted to a concentration of 5×10^3/μL using PBS. Monoclonal antibodies (20 L) were added to 100 L of the adjusted whole-blood suspension. The suspension was incubated for 30 minutes at 4°C and vortexed every 5 minutes. After incubation with antibodies, the erythrocytes were lysed by an erythrocyte lysing reagent (Orthomune™; Ortho Diagnostic Systems, Raritan, NJ) by incubating the suspension for 10 minutes in a tumbler. The suspension was washed twice by centrifugation at 400*g* for 5 minutes with 2 mL PBS containing 0.5% BSA and 0.1% NaN_3. The pellet was resuspended in 400 L PBS/BSA/NaN_3 and analyzed within 30 minutes.

TABLE 1. Antibody panel of the multiparametric flow cytometry analysis with determination of the data columns for the CLASSIF1 program

	FITC	PE	PerCP/PE-Cy5
1	IgG_2	IgG_1	IgG_2
2	CD45	CD14	CD3
3	CD33	CD34	CD13
4	CD38	CD34	HLA-DR
5	CD15	CD14	CD16
6	CD4	CD14	CD64
7	CD7	CD56	HLA-DR
8	CD11c	CD11b	HLA-DR
9	Myeloperoxidase		

Monoclonal Antibodies

For three-color staining, directly conjugated monoclonal antibodies were added simultaneously to the cells, and the suspension was incubated and washed as described above. The antibodies were prediluted to ratios between 1:2 and 1:5 with PBS after appropriate titration experiments in the FACScan flow cytometer. No fixative reagents were used.

The following FITC-conjugated (FITC) monoclonal antibodies were used: CD45 (2D1, γ1-isotype; Becton Dickinson, Heidelberg, Germany), CD33 (WM-54, γ1-isotype; DAKO Diagnostica, Hamburg, Germany), CD38 (T16, γ1-isotype; Dianova-Immunotech, Hamburg, Germany), CD15 (HMA, μ-isotype; Becton Dickinson), CD4 (SK3, γ1-isotype; Becton Dickinson), CD7 (4H9, γ2a-isotype; Becton Dickinson), CD11c (KB90, γ1-isotype; DAKO). For the second fluorescence, R-phycoerythrin (R-PE) conjugated antibodies were added: CD14 (MoP9, γ2b-isotype; Becton Dickinson), CD34 (8G12, γ1-isotype; Becton Dickinson), CD56 (My31, γ1-isotype; Becton Dickinson), CD11b (D12, γ2a-isotype; Becton Dickinson). For the third fluorescence, antibodies conjugated by PerCP or R-PE covalently labeled with cyanine 5 (PE-Cy5) were added: CD13 (WM-47, γ1-isotype; DAKO), CD3 (SK7, γ1-isotype; Becton Dickinson), HLA-DR (L243, γ2a-isotype; Becton Dickinson), CD16 (3G8, γ1-isotype; Caltag Laboratories, San Francisco, CA), CD64 (32.2, γ1-isotype;° Caltag Laboratories). MPO-7 (DAKO) was used for intracellular analysis of myeloperoxidase after cell permeability had been increased by Ortho Permeafix™ (Ortho Diagnostic Systems; Dianova) [9]. The antibody panel is shown in Table 1.

Flow Cytometry

Flow cytometric data acquisition was performed on a FACScan (Becton Dickinson) by the FACScan Lysis II software. The instrument setup was standardized threefold using lymphocytes from normal persons according to AUTOcomp settings

TABLE 2. Cell parameter differences between PBL, BM, GBL, and GBM samples

Number	Antibody (Ab) parameters	Ab –/+ cells of quadrant analysis[a]	Cells	PBL[b]	BM	GBL	GBM
1	Ab ratio CD38/34 (CD34/38)	– –	lymphocyte	0	0	+	+
2	Ab ratio CD13/33 (CD33/13)	– –	lymphocyte	0	+	+	–
3	Ab content IgG_2 (IgG_1/IgG_2)	+ +	lymphocyte	0	+	+	+
4	Ab ratio IgG_2/IgG_1 (IgG_1/IgG_2)	+ +	lymphocyte	0	0	–	0
5	Percent CD16 (CD14/16)	–	monocyte	0	0	0	+
6	FSC CD16 (CD14/16)	–	monocyte	0	0	0	–
7	Ab ratio CD13/34 (CD34/13)	+ –	monocyte	0	0	+	+
8	Ab content CD45 (CD45/14)	–	granulocyte	0	+	0	0
9	Ab surface density CD45 (CD45/14)	–	granulocyte	0	+	+	+
10	Ab content CD14 (CD45/14)	–	granulocyte	0	+	+	+
11	FSC CD15 (CD15/14)	–	granulocyte	0	+	+	0
12	Percent HLA-DR (CD34/HLA-DR)	–	granulocyte	0	0	–	0
13	FSC CD16 (CD14/16)	–	granulocyte	0	+	+	+
14	Ab content CD11c (CD11c/11b)	– –	granulocyte	0	0	–	0
15	Ab content CD3 (CD14/3)	– –	granulocyte	0	+	+	+
16	Ab ratio CD3/45 (CD45/3)	– –	granulocyte	0	–	0	0
17	Percent CD34/HLA-DR (CD34/HLA-DR)	– –	granulocyte	0	0	–	0
18	Ab ratio CD16/15 (CD15/16)	– –	granulocyte	0	–	+	–
19	FSC CD15 (CD15/14)	+	granulocyte	0	+	0	0
20	Ab ratio CD13/34 (CD34/13)	+ –	granulocyte	0	+	+	+

[a]Cellular antigen expression, e.g., no. 1: – – = Ab ratio of CD38/CD34 double-negative cells; no. 5: – = percent CD16-negative cells in CD14/CD16 histogram.

[b]The sequences of 0, + ,– signs constitute the reference sample classification masks of the PBL, BM, GBL, and GBM samples. 0 = the respective parameter for a sample should be within the 15th and 85th percentiles of the value distribution of all PBL samples. + = the values are above the 85th percentile; – = the values are below the 15th percentile of the PBL samples. Unknown samples are classified according to the highest positional coincidence with any of the reference classification masks.

(Becton Dickinson), using standardized fluorescent beads (Fluorospheres [Rainbow beads], DAKO) and CD4 (SK3, γ1-isotype; Becton Dickinson), CD8 (SK1, γ1-isotype; Becton Dickinson), and CD3 (SK7, γ1-isotype; Becton Dickinson) staining from lymphocytes of normal donors. The linear FSC and orthogonal SSC in combination with four-decade logarithmic FITC, PE, and PerCP/PECy5 fluorescence signals were collected for 20,000 cells and stored in list mode data files. Analysis was performed using Lysis II software as well as CLASSIF1 software.

CLASSIF1 Analysis

The CLASSIF1 program system operates in personal computers using MS-DOS 6.2 or WINDOWS 3.1 (for details see [10]). Flow cytometric FSC 1.0 or FSC 2.0 list-mode files of up to eight parameters were processed by the DATLYS procedure, which generates three-, two-, and one-parameter histograms. Two-parameter histograms were obtained through gated or ungated projection of the multidimensional list mode on any wanted coordinate plane and evaluated by a multiwindow calculation procedure, CALC. Program CALC determined absolute and percent cell frequency, mean FSC, SSC, SSC/FSC ratio, mean antibody intensity in FITC, PE, PerCP, PECy5, as well as FITC/PE, PE/PerCP or PECy5, FITC/PerCP or PECy5 fluorescence ratios, and FITC, PE, PerCP relative antibody surface density for each evaluation window. The antibody surface density was obtained as ratio of fluorescence per square root of FSC, assuming that FSC represents a cell volume–like signal. The resulting numerical data were automatically introduced into task-specific databases. Procedure LEARN determined a lower and an upper percentile (e.g., 15 and 85%) for the peripheral blood values of each database column that served as a reference. Database column values of peripheral blood and other samples were then transferred into triple matrix characters by assigning "0" to values between both percentiles, "+" to values above the upper, and "–" to values below the lower percentiles. The most frequent triple-matrix character was introduced database column per database column into classification reference masks for each classification state (Table 2, rightmost four columns). The triple matrix pattern of each sample of the database was then classified according to the highest positional coincidence with one of the classification reference masks. By repeated iterations, a triple-matrix reference mask for each classification state was optimized from the learning set database in such a way that the highest overall distinction was obtained for each classification state in the confusion matrix. The confusion matrix (Table 3) contained the different preparation methods on the ordinate and the classification of the flow cytometric database on the abscissa. Database columns that did not improve the result were omitted during the iterations because their presence in the classification mask deteriorated the overall classification result.

The sum of the percentages in the horizontal lines of the confusion matrices (Table 3) may be higher than 100% when some samples are classified into more than one classification state. This sum does not, however, affect the values of the correct classifica-

TABLE 3. Confusion matrix for the classification of PBL, BM, GBL, and GBM samples

Clinical diagnosis	Patient number	Flow classification			
		PBL	BM	GBL	GBM
PBL	6	**100.0**	0.0	0.0	0.0
BM	6	16.7	**83.3**	0.0	0.0
GBL	6	0.0	0.0	**100.0**	16.7
GBM	6	0.0	16.7	0.0	**83.3**

Confusion matrix (% patients); database: G7LEARN.B14.

Values in bold provide the basis for calculating ARI and AMI.

ARI = 91.7%. The ARI is calculated as sum of the diagonal values of the confusion matrix divided by the number of classification states. The ARI is a standardized indicator for the rapid comparison of different confusion matrices.

AMI = 1.04 (acceptable range: 1.0–1.2). Differential classification percentile range = 15–85%. Samples may be classified into more than one classification state. The sum of the percentages in the horizontal lines of the confusion matrix is then higher than 100%. This does not affect the classification values in the diagonal of the confusion matrix. For the determination of the AMI, all values in the horizontal lines are added and divided by the total number of boxes in the confusion matrix (e.g., 4 × 4 = 16 in Table 3). The AMI is a standardized indicator for multiple classifications. If the AMI is 1.1, 1.2, 1.33, or 1.5, every 10th, 5th, 3rd, or 2nd sample is classified twice. Therefore, ARIs are only acceptable for AMIs between 1.0 and 1.2.

tion in the boxed diagonal of the confusion matrices; the sum of the percentages in the diagonal line of the confusion matrix is 100% for a perfect classification. The speed of the triple-matrix classification program is high because learning the new database columns takes only minutes on an Intel PC (80486, 90 MHz) and classification of unknown samples takes only milliseconds.

CLASSIF1 classification

Samples were taken from 6 patients, and 284 flow cytometric measurements were performed after density gradient centrifugation (GBM n=6, GBL n=6) or erythrocyte lysis (BM n=6, PBL n=6) of bone marrow or leukemic peripheral blood samples. Three automatically self-adjusting light-scatter gates were determined for lymphocyte, monocyte, and granulocyte cell clusters within the FSC and against the SSC histogram of each patient sample (Fig. 1). Three immunofluorescence histograms (green/orange, green/red, orange/red fluorescence) were then calculated for each scatter gate, yielding 74 data columns each (i.e., a total of 3 × 74 = 222 data columns for the three-color measurements; see Fig. 2 for an example of FITC/PE/PerCP staining of lymphocytes).

The immunofluorescence histograms were evaluated for antibody-positive and -negative cells and analyzed by quadrant analysis. The threshold between antigen-positive and -negative cells was set at 31.2% of the maximum fluorescence channel for each fluorescence color. Nine different three-color measurements (CD45/14/3, CD33/34/13, CD38/34/HLA-DR, CD15/14/16, CD4/14/64, CD7/56/HLA-DR, CD11c/11b/HLA-DR, MPO, $IgG_2/IgG_1/IgG_2$) were evaluated per patient sample, providing a total of 9 × 222 = 1998 data columns for data classification with the CLASSIF1 program. The optimal light-scatter evaluation gates for lymphocytes, monocytes, and granulocytes were automatically adjusted for each measurement.

The aim of the CLASSIF1 analysis was to examine the influence of density gradient centrifugation and erythrocyte lysis with regard to systematic differences for leukemic peripheral blood or bone marrow samples. A separate analysis was calculated for each cell type and for all cell types together. The data obtained from peripheral blood prepared by erythrocyte lysis served as reference data to be compared with bone marrow cells and peripheral blood cells prepared by density gradient centrifugation and with bone marrow prepared by erythrocyte lysis.

RESULTS

Acute myelogenous leukemia was correctly diagnosed by Lysis II analysis in all patients using density gradient centrifugation and erythrocyte lysis preparations. Lysis II analysis did not, however, provide any clues to the relevant parameter differences between the different sample preparations. These differences became clear only after the CLASSIF1 data analysis. According to the CLASSIF1 classification, most of the chosen parameters were parameters of mean antigen intensity, antibody surface density, or fluorescence mean ratio (Table 2). Differences of percent contribution of cell populations were only rarely obtained. Blood and bone marrow cell suspensions prepared by density gradient centrifugation or erythrocyte lysis were equally well recognized, with single case recognition frequencies of 100 and 83.3% of all samples (i.e., the different samples were readily distinguishable; Table 3).

Separate analysis of the lymphocyte and granulocyte gates (Table 4) showed reasonable results for the recognition of the different samples with an average recognition index (ARI) of 62.5 and 83.3%, respectively, at an average multiplicity index (AMI) of 1.08. The analysis of the monocyte gate provided insufficient classification information with an ARI of 70.8% and an AMI of 1.38 (Table 4). These results show that the relevant information for the computer-assisted classification was mainly localized in the lymphocyte and granulocyte gates (Fig. 1).

In general, the parameters of antigen intensity and antibody surface density of samples prepared by density gradient centrifugation were higher compared with erythrocyte lysis. Interestingly, cell depletion by density gradient centrifugation as expressed by percent contribution of all cell populations was not frequently chosen as a relevant parameter to distinguish between the different preparative methods (Table 2). The relevant information with regard to antibody fluorescence, in contrast, was chosen in 13 (65%) of the 20 classification parameters.

The isotype measurements were classified only two times (parameters 3 and 4, Table 2). Both parameters were higher in the density gradient centrifugation when compared with the preparations obtained by erythrocyte lysis. According to these findings, IgG_2 binding was increased by density gradient centrifugation, especially if PE-conjugated antibodies were used.

DISCUSSION

In previous studies, antigen expression by leukocytes prepared by density gradient centrifugation or erythrocyte lysis had pri-

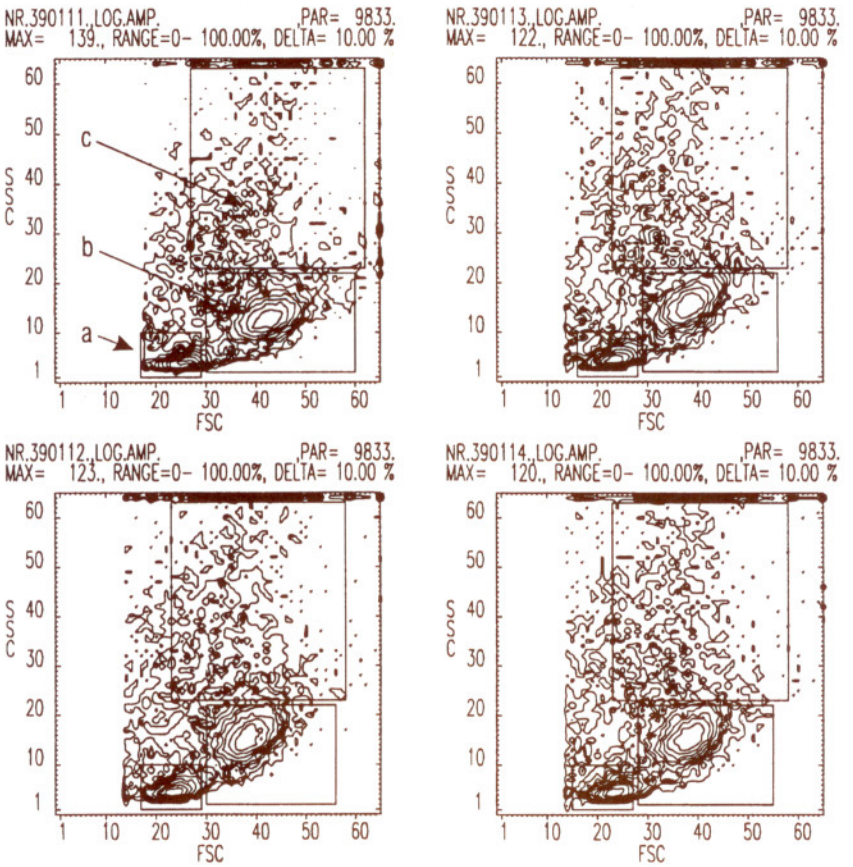

FIGURE 1. Two-parameter contour plots of the lymphocyte (a), monocyte (b), and myeloblast/myelocyte (c) clusters of an AML bone marrow sample prepared by erythrocyte lysis. The forward (FSC) and side (SSC) scatter evaluation gates were automatically set by the CLASSIF1 program. The amplitude scale spans three logarithmic decades. Contour lines are plotted at 10% intervals from the maximum logarithmic channel content downwards. Sample size is 9.833 cells.

marily compared blood from normal donors used for immunophenotyping of normal lymphocytes [3,5,6]. A loss of lymphocyte subpopulations and an enrichment of natural killer cell populations are common in samples prepared with density gradient centrifugation. Interestingly, contamination in newborns of lymphocyte populations with immature erythrocytes gave rise to the misinterpretation that an undifferentiated lymphocyte population in the lymphocyte gate might be present [11]. An occasional loss of leukemic blasts was also observed by density gradient centrifugation [12]. Erythrocyte lysis advantageously avoids the difficulties of density gradient centrifugation for the preparation of mononuclear cells. The practice of leukocyte staining followed by erythrocyte lysis is now established [13]. In one study, almost all lytic reagents examined gave equivalent results with regard to lymphocyte numbers and the proportions of lymphocyte subpopulations [3]. However, nucleated erythroblasts and granulocytes are not lysed and can therefore be additionally analyzed by flow cytometry.

Tamul *et al.* [14] compared the effects of FH separation and whole blood lysis in patients suffering from various hematologic disorders by using two-color flow cytometric immunophenotyping. The authors discriminated eight antigens that demonstrated significantly different staining characteristics after density gradient centrifugation and whole blood lysis: FITC-labeled CD2, CD3, CD5, CD22, CD34, and PE-labeled CD11c, CD20, and CD25. Unfortunately, neither the isotypes of antibodies nor the analyzed histogram regions were demonstrated; thus, a comparison with the present results is difficult. Moreover, with the exception of CD34, a significant difference of positive percentage frequency was a result of placement of the quadrant markers based on isotype control antibodies. In seven cases (two with acute leukemia, one with small lymphocytic leukemia, four with hairy cell leukemia) a selective loss of CD34-positive cells was seen after density gradient centrifugation. Unfortunately, the cell pellet was not reinvestigated to check for CD34-positive cells.

Islam et al. [15] recently published a study comparing the two preparation methods in blood samples obtained from healthy adults. The samples were stored overnight and analyzed with multicolor flow cytometric immunophenotyping. CD4, CD3, CD8, CD19, CD20, CD25, and CD56 antibodies were conjugated

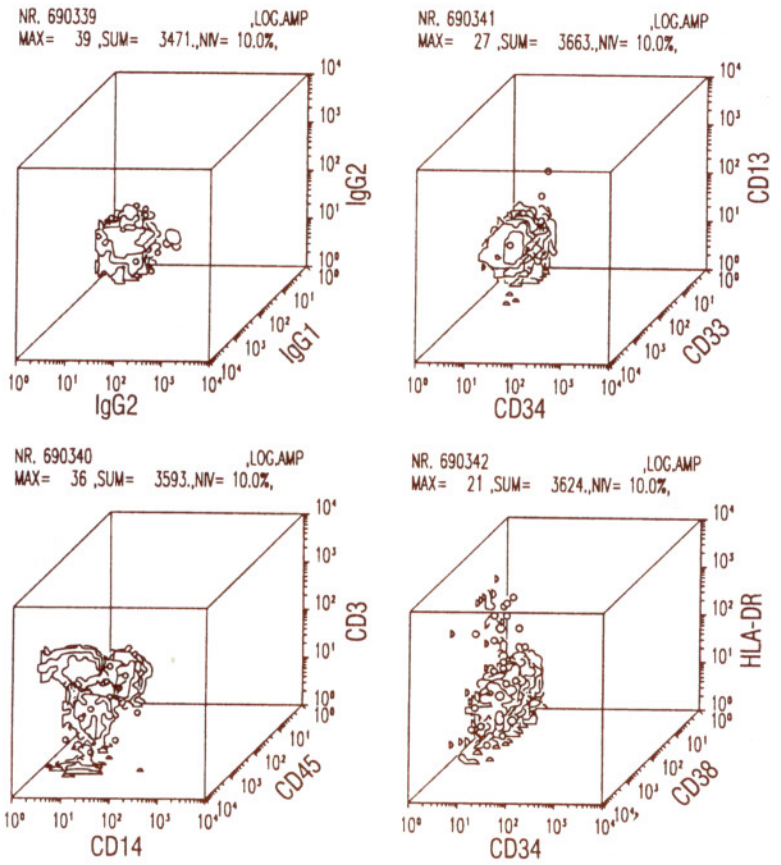

FIGURE 2. Three-parameter contour plot of a gradient prepared blood leukocyte AML sample following immunostaining by FITC/PE/PerCP-Cy5 directly labeled antibodies. The contour lines are plotted at the 10% level of the maximum logarithmic channel content of the three decade amplitude scale. Sample size as in Figure 1.

with different fluorochromes and analyzed with different staining panels. Surprisingly, the authors could not detect the well-known loss of CD8-expressing T cells after overnight storage and density gradient centrifugation; instead, they found a significant decrease of absolute CD8 intensity after density gradient centrifugation. Using quantitative immunophenotyping, an increase of absolute fluorescence intensities was found in samples prepared with density gradient centrifugation when compared with erythrocyte lysis. Furthermore, the same antibodies conjugated to different fluorochromes gave different fluorescence intensity results and the same antibody conjugated to the same fluorochrome gave differences in fluorescence intensity when used in three-color analysis in combination with various other antibodies.

TABLE 4. Recognition of PBL, BM, GBL, and GBM samples by the CLASSIF1 program

	Percent CLASSIF1 recognition					
Cells	PBL	BM	GBL	GBM	ARI (%)	AMI
Lymphocytes	**100**	**33**	**66**	**50**	**62.5**	**1.08**
Monocytes	100	83	50	50	70.8	1.38
Granulocytes	**100**	**83**	**83**	**66**	**83.3**	**1.08**
Lymphocytes + monocytes + granulocytes	**100**	**83**	**100**	**83**	**91.7**	**1.04**

For all classifications, n=6.

Values in bold are considered reasonable, while the values for monocytes are considered insuffient for classification.

In this investigation, preparation by erythrocyte lysis served as a reference method (Table 3). As mentioned above, cells prepared by density gradient centrifugation generally showed higher antigen expression (Table 2). Density gradient centrifugation and washing procedures might be responsible for the increase of antigen expression by removing parts of the cell surface layer (glycocalix). In addition, accessibility of antigens might be improved by the fact that the cells remained unfixed. Interestingly, density gradient centrifugation led to an increase of IgG_2 binding, but the general increase of antigen expression by this preparative procedure is not related to IgG conjugation because the CLASSIF1 program did not reveal a comparable increase of other PE and PerCP/PE-CY5 conjugates.

Peripheral blood and bone marrow samples obtained by erythrocyte lysis or density gradient centrifugation preparation were recognized with a single-case recognition index of 100 and 83.3% (Table 3). Thus far, the results obtained refer only to patients with acute myelogenous leukemia. Further studies are necessary to ascertain whether preparation by density gradient centrifugation will lead to an increase of antigen intensity in other hematologic disorders (such as acute lymphoblastic leukemia or minimal residual disease) [16].

The Lysis II extraction of the characteristic values of two-dimensional or multidimensional cell clusters, such as means, coefficients of variation, modes, or medians, is not sufficient to obtain distinct information. Progress toward a generally and routinely applicable automated list-mode files classification program for flow cytometry has remained limited [17–19] because of the relatively complex algorithms. As demonstrated by the results presented in this paper, the newly developed CLASSIF1 analysis [10], in contrast, was able to extract adequately the needed information from nearly 2000 data columns in a short period of time. The content of only 26 database columns is sufficient to discriminate an unknown leukemic peripheral blood or bone marrow sample obtained by preparation with density gradient centrifugation or erythrocyte lysis. Based on the established CLASSIF1 program classifiers, it is seemingly possible to differentiate patients with acute myelogenous leukemia from patients with other forms of leukemia by this same classification procedure. A series of standardized classifiers to compare automatically various leukemias and lymphomas seems reasonable for the future [17]. The advantage of such defined classifiers is that they are principally independent of the type of flow cytometer, provided that it is long-term calibrated by stable fluorescent beads, that it is capable of measuring the light scatter and fluorescence signals, and that the antibodies used identify the same antigens as used for the classification.

In conclusion, the objective of the present study was to investigate systematic differences of peripheral blood and bone marrow samples prepared by erythrocyte lysis and density gradient centrifugation using computerized list-mode data files analysis. The CLASSIF1 program, by discriminating the different suspensions, was able to detect those antigen parameter patterns that could not be obtained using Lysis II. Density gradient centrifugation led to a clear-cut increase of antigen expression and antibody fluorescence intensity, and it improved the staining of antibodies against weak antigens. The relevant information for the computer-assisted classification was obtained in the lymphocyte and granulocyte gates. For a correct classification procedure, no significant difference was noted between samples obtained from bone marrow or those from leukemic peripheral blood. Common cell depletion by density gradient centrifugation did not substantially influence the classification results in the case of acute myelogenous leukemia.

REFERENCES

1 Terstappen LWMM, Safford M, Könemann S, Loken MR, Zurlutter K, Büchner Th, Hiddemann W, Wörmann B: Flow cytometric characterization of acute myeloid leukemia. Part II. Phenotypic heterogeneity at diagnosis. Leukemia 5:757, 1991

2 Drexler HG, Thiel E, Ludwig W-D: Acute myeloid leukemias expressing lymphoid-associated antigens: diagnostic incidence and prognostic significance. Leukemia 7:489, 1993

3 Carter PH, Resto-Ruiz S, Washington GC, Ethridge S, Pallini A, Vogt R, Waxdal M, Fleisher T, Noguchi PD, Marti GE: Flow cytometric analysis of whole blood lysis, three anticoagulants, and five cell preparations. Cytometry 13:68, 1992

4 National Commitee for Clinical Laboratory Standards: Clinical applications of flow cytometry: quality assurance and immunophenotyping of peripheral blood lymphocytes; tentative guideline. NCCLS document H42-T (ISBN 1-56238-155-5), Villanova, PA, 1992

5 Serke S, Huhn D: An all leucocyte whole blood lyse method for multiparameter flow-cytometry. Clin Lab Haematol 15:275, 1993

6 De Paoli P, Reitano M, Battistin S, Castiglia C, Santini G: Enumeration of human lymphocyte subsets by monoclonal antibodies and flow cytometry: a comparative study using whole blood or mononuclear cells separated by density gradient centrifugation. J Immunol Methods 72:349, 1984

7 Bennet JM, Catovsky D, Daniel MT, Flandrin G, Galton DAG, Gralnick HR, Sultan C: Proposals for the classification of the acute leukemias. Br J Haematol 33:451, 1976

8 Jackson AL, Warner NL: Preparation and analysis by flow cytometry of peripheral blood leucocytes. In: NR Rose, H Friedman, JL Fahey (eds) Manual of Clinical Laboratory Immunology. American Microbiology Association, Washington, D.C., 1986, p. 226

9 Pizzolo G, Vincenci C, Nadali G, Veneri D, Vinante F, Chilosis M, Basso G, Connely MC, Jannosy G: Detection of membrane and intracellular antigens by flow cytometry following ORTHO permeafix fixation. Leukemia 8:672, 1994

10 Valet G, Valet M, Tschöpe D, Gabriel H, Rothe G, Kellermann W, Kahle H: White cell and thrombocyte disorders. Standardized, self-learning flow cytometry list mode data classification with the CLASSIF1 program system. Ann NY Acad Sci 677:233, 1993

11 Slade HB, Greenwood JH, Hudson JL, Beekman RH, McCoy JP, Schwart SA: Spurious lymphocyte phenotypes by flow cytometry from mononuclear cells prepared by Ficoll-Hypaque. Pediatr Res 21:318A (abstr), 1987

12 McCoy JP, Carey JL, Krause JR: Quality control in flow cytometry for diagnostic pathology. Am J Clin Pathol 93 (Suppl. 1):27, 1990

13 Ashmore LM, Shopp GM, Edwards BS: Lymphocyte subset analysis by flow cytometry. Comparison of three different staining techniques and effects of blood storage. J Immunol Methods 188:209, 1989

14 Tamul KR, Schmitz JL, Kane K, Folds JD: Comparison of the effects of Ficoll-Hypaque separation and whole blood lysis on results of immunophenotypic analysis of blood and bone marrow samples from patients with hematologic malignancies. Clin Diagn Lab Immunol 2:337, 1995

15 Wörmann B, Könemann S, Humpe A, Safford M, Zurlutter K, Schreiber K, Piechotka, Büchner Th, Hiddemann W, Terstappen LWMM: Detection of residual leukemic cells in AML. In: W Hiddemann (ed) Haematology and Blood Transfusion, Springer-Verlag, Berlin, 1992, p. 196

16 Islam D, Lindberg AA, Christensson B: Peripheral blood cell preparation influences the level of expression of leuocyte cell surface markers as assessed with quantitative multicolor flow cytometry. Cytometry 22:128, 1995

17 Bierre P, Mickaels R, Thiel D: Multidimensional visualization and autoclustering of flow cytometric data. Cytometry 12 (Suppl. 5):64, 1991

18 Frankel DS, Frankel SL: Real time network for flow cytometry analysis. Cytometry 12 (Suppl. 5):63, 1991

19 Verwer BJH, Terstappen LWMM: Automatic lineage assignment of acute leukemias by flow cytometry. Cytometry 14:862, 1993