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Original Articles

Automated Classification of Patients With Chronic Lymphocytic Leukemia and Immunocytoma From Flow Cytometric Three-Color Immunophenotypes

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The goal of this study was the discrimination between chronic lymphocytic leukemia (B-CLL), clinically more aggressive lymphoplasmocytoid immunocytoma (LP-IC) and other low-grade non-Hodgkin's lymphomas (NHL) of the B-cell type by automated analysis of flow cytometric immunophenotypes CD45/14/20, CD4/8/3, kappa/CD19/5, lambda/CD19/5 and CD10/23/19 from peripheral blood and bone marrow aspirate leukocytes using the multiparameter classification program CLASSIF1.

The immunophenotype list mode files were exhaustively evaluated by combined lymphocyte, monocyte, and granulocyte (LMG) analysis. The results were introduced into databases and automatically classified in a standardized way. The resulting triple matrix classifiers are laboratory and instrument independent, error tolerant, and robust in the classification of unkown test samples. Practically 100% correct individual patient classification was achievable, and most manually unclassifiable patients were unambiguously classified. It is of interest that the single lambda/CD19/5 antibody triplet provided practically the same information as the full set of the five antibody triplets. This demonstrates that standardized classification can be used to optimize immunophenotype panels. On-line classification of test samples is accessible on the Internet: http://www.biochem.mpg.de/valet/leukaem1.html

Immunophenotype panels are usually devised for the detection of the frequency of abnormal cell populations. As shown by computer classification, most the highly discriminant information is, however, not contained in percentage frequency values of cell populations, but rather in total antibody binding, antibody binding ratios, and relative antibody surface density parameters of various lymphocyte, monocyte, and granulocyte cell populations. Cytometry 30:275–288, 1997. © 1997 Wiley-Liss, Inc.

Key terms: flow cytometry; chronic lymphocytic leukemia; immunocytoma; automated classification; artificial intelligence

Chronic lymphocytic leukemia (CLL) and immunocytoma (IC) represent two separate entities of lymphocytic low-grade non-Hodgkin's lymphomas according to the Kiel classification (4,15,19,27). This distinction is predominantly based on morphological and immunohistochemical differences in lymph node analysis e.g., the presence of periodic acid-Schiff (PAS)-positive intranuclear bodies (''Dutcher bodies''), cytoplasmic immunoglobulin, and characterization of cells with monoclonal antibodies and cytogenetic methods. Characteristic chromosomal aberrations occur, e.g., in B-CLL (13,17,19).

CLL and the lymphoplasmocytoid variant of immunocytoma (LP-IC) have been defined as small lymphocytic lymphoma (SLL) and SLL/plasmocytoid variant (SLL/p) by the REAL classification, whereas the former lymphoplasmocytic IC of the Kiel-classification was re-defined as lymphoplasmocytoid immunocytoma by the REAL classification. Thus, from the three former entities of the Kiel-classification two have been reclassified as small lymphocytic lymphomas (SLL and SLL/p), whereas the third entity IC has been renamed (14).

The question arose whether the immunomorphological differences are paralleled by different clinical disease characteristics. Long-term survival analysis revealed significant differences between B-CLL and LP-IC in a prospective multicenter observation study conducted by the Kiel Lymphoma Study Group (9). The pseudofollicular form in B-CLL and the IC are more favorable histological subtypes than the lymphoplasmocytoid form (LP-IC) of immunocy-

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tomas. Thus, the distinction between B-CLL and LP-IC is of practical clinical interest.

The flow cytometric determination of multicolor immunophenotypes should in general enhance the discrimination potential between various antigenic patterns in hematopoietic malignancies. Substantial problems of understanding and interpreting visual and calculated results exist, however, due to the high number of simultaneously measured parameters and due to the multiple measurements.

Several proposals for the automated information extraction from multiparameter flow cytometric measurements have been made. Neural networks (3,10,11,20,22), principal component (18), cluster (7,24,29,33), or discriminant (20) analysis, hierarchical classifiers (6), classification and regression trees (CART, 1), as well as knowledge based systems (8,30), statistical classifiers (23), or fuzzy logic (20), seem promising approaches. No easily adaptable classification procedures for routine applications have, however, so far resulted from these efforts.

The recent development of triple matrix classifiers (12,28,31,32) has substantially facilitated the separate or combined classification of any kind of cytometric and other clinical or experimental multiparameter data. High numbers of database columns can be handled on standard personal computers in short time periods and standardized; i.e., laboratory- and instrument-independent classifiers are obtained as they are required for international consensus formation. Such consensuses are an essential prerequisite for the generalized routine practice of cytometric assays.

The goal of the present study concerned the discrimination between B-CLL and LP-IC as well as between different low- aand high-grade lymphomas of the B-cell type. It was of particular interest to correlate the morphological differences with immunological cell parameters. The clinical diagnosis was determined prior to computer learning by morphology, by visual classification of three-color leukocyte immunophenotyping and by clinical data. The morphological information was obtained by histological examination of lymph node or spleen specimens.

MATERIALS AND METHODS

Patients and Cell Sample Characteristics

The patients of the *learning set* were recruited between May 1992 and October 1995. They were all fully characterized prior to antimalignant therapy in the following way:

Diagnostic specimens included bone marrow aspirates and peripheral blood smears as well as bone marrow, lymph node, or spleen examination. All patients with B-CLL and LP-IC had an absolute lymphocytosis ($>15 \times 10^3$/ml) in their peripheral blood and a bone marrow infiltration. Leukemic disease was defined by lymphocytes or lymphoma cells exceeding 15×10^3/ml in peripheral blood (26). The differential counts of both groups (B-CLL and LP-IC) were not significantly different.

Bone marrow trephines were performed by bone penetration with a Jamshidi needle from the posterior iliac crest by the standard technique (16). Bone marrow aspirates were obtained before or after biopsy by repositioning the Jamshidi needle through the same skin incision into a different place of the iliac crest; 0.2–0.5 ml bone marrow was withdrawn into a syringe containing only preservative-free heparin. Smears or touch preparations of the aspirate were immediately performed and air-dried. Peripheral blood was obtained by venipuncture of the V. cubitalis.

The histological results were classified according to the Kiel classification (19). All patients were thoroughly clinically staged. Staging results for B-CLL and LP-IC patients were classified in parallel according to the Ann Arbor (5), Rai (21), and Binet (2) staging systems. Flow cytometric immunophenotyping (details see below) was performed in all cases with particular emphasis on light chain restriction, detection of CD5, and presence or absence on monotypical B cells, defined by simultaneous CD45/CD20, CD5/CD19, and CD23/CD19 staining.

Lymphomas without CD5 expression but with characteristics of IC were classified as lymphoplasmocytic lymphoma. Such cases were excluded from the study. Among 40 qualified patients, the diagnosis of B-CLL was established in 15 (5 females and 10 males, median age 68 years, range 51–73 years), and of LP-IC in 14 (4 females and 10 males, median age 60 years, range 45–69 years) patients, whereas the remaining 11 cases where classified as lymphocytic lymphomas without further specification. The cytomorphological and immunocytological examination of bone marrow and peripheral blood samples and the additional histopathological examination of bone marrow samples were performed independently by two different observers. Bone marrow infiltration was verified by either positive trephine histology or by both positive cytomorphologic and positive immunocytologic investigation (25).

Additional lymphoma cases as well as patients with unspecified leukocytosis were included in the learning set after informed patient consent: 8 patients suffering from hairy cell leukemia (HCL), one patient with a mantle cell (centrocytic) lymphoma (CCL), two patients with centroblastic-centrocytic lymphoma (CB-CCL), one patient with a high-grade centroblastic lymphoma (CBL) and one patient with a undifferentiated high-grade B-cell lymphoma (LGR). The populations characteristics for these patients differed not significantly from the above described patient group. Furthermore, 12 patients (median age 55 years, range 45–67 years) submitted to the outpatient department with unspecified leukocytosis with increased granulocyte and lymphocyte numbers were included into the learning set as unspecified leukocytosis cases. In none of these patients was a hematological disease detected despite thorough clinical and laboratory investigations. Blood and bone marrow samples were taken after informed patient consent from 15 healthy volunteers (8 males and 7 females), selected as bone marrow donors for compatible or semicompatible allografts. The age distribution of these donors ranged from 20–45 years with an average of 29 years. Except for birth control pills, none of the donors was taking any medication.

The test set of patients was unknown to the classifiers and consisted of measurements from 11 healthy subjects

(see below) and from 4 B-CLL and 2 LP-IC cases. In addition, 19 patients with partially unclear diagnosis, such as unproven lymphoma, were investigated as a second test set to assess the capability of the classifiers to classify samples with uncertain clinical diagnosis. This group included confirmed lymphomas, whose correct subtype could not be established despite lymph node specimen examination and the patients were clinically classified as ''lymphocytic lymphoma.''

Immunophenotype Assays

Leukocyte concentrations in peripheral blood and bone marrow samples were adjusted with PBS to 5×10^3 cells/ml. Monoclonal antibodies (10–20 µl/100 µl diluted blood) were added in pretitered concentrations followed by vortexing and 30-min incubation at 0°C with vortexing every 10 min. A quantity of 2 ml Ortho-Lyse (Ortho, Heidelberg, Germany) was added to the sample with immediate vortexing followed by 10min incubation at 0°C for erythrocyte lysis. The sample was then washed twice with 4 ml phosphate-buffered saline (PBS) by centrifugation for 5 min at 400*g*, the supernatant was discarded and the sediment resuspended in 2 ml PBS and 0.4 ml P was BS/0.5% bovine serum albumin (BSA)/0.1% Na azide and kept in the dark on ice until the flow cytometric measurement.

Antibody Panel

Fluorescein isothiocyanate (FITC) coupled monoclonal CD10 (SS2/36, IgG1 isotype; DAKO), CD38 (T16, IgG1 isotype; Immunotech, Hamburg, Germany), CD45 (T29/33, IgG1 isotype; DAKO Diagnostika, Hamburg, Germany), CD103 (Bly7, IgG1 isotype; DPC Biermann, Bad Nauheim, Germany), kappa (rabbit anti-human F(ab′)2, polyclonal; DAKO), lambda (rabbit anti-human F(ab′)2, polyclonal; DAKO), IgM (rabbit anti-human F(ab′)2, polyclonal; DAKO), FMC-7 (IgG1 isotype; Immunotech), as well as R-Phycoerythrin (PE) coupled CD8 (SK1; IgG1 isotype, Becton-Dickinson, Heidelberg, Germany), CD11c (S-HCL-3, IgG1 isotype; Becton-Dickinson), CD14 (TUEK 4, IgG1 isotype; DAKO), CD19 (4G7, IgG1 isotype; Becton-Dickinson), CD 23 (EBVCS-5, IgG1 isotype, Becton-Dickinson), IgD (goat anti-human F(ab′)2, polyclonal; Southern Biotechnology, Birmingham, USA), B-B4 (IgG1 isotype; DPC Biermann) and furthermore Peridinin Chlorophyll A Protein (PerCP) CD3 (SK7; IgG1 isotype, Becton-Dickinson) and CD20 (L27, IgG1 isotype; Becton-Dickinson) as well as Phycoerythrin/Cyanine 5 (PE-Cy5) coupled CD5 (5.D7, IgG1 isotype; Caltag Laboratories, San Francisco, CA) and CD19 (HD37, IgG1 isotype; DAKO) were used.

Antibody combinations for supposed low-grade non-Hodgkin's lymphomas were CD45/14/20, CD4/8/3, kappa/CD19/5, lambda/CD19/5, CD10/23/19, IgG2/IgG2/IgG1, sIgM/sIgD/CD19, CD38/BB-4/CD19, CD10/38/19, CD103/11c/19, sIgM/sIgD/CD38 while CD45/14/20, CD4/8/3, kappa/CD19/5, lambda/CD19/5, CD10/23/19, IgG1/IgG2/IgG1, IgG2/IgG1/IgG1, IgG1/IgG1/IgG1, CD103/11c/19, sIgM/CD38/CD19 combinations were used for supposed B-CLL. The first five typing triplets were identical for both groups and could therefore be used for comparative automated classifications.

Flow Cytometry

Cell samples were processed within 2 h after specimen collection. Analysis was performed on a FACScan (Becton-Dickinson) analytical flow cytometer. List mode data were acquired by the Lysis II software (Becton-Dickinson). The cellular forward (FSC) and sideward scatter (SSC) signals as well as the fluorescence of cell membrane bound FITC, PE, PerCP, or PE-Cy5-labeled antibody were determined following illumination of the cells in the focal spot of a 15mW air cooled argon-ion laser at 488 nm in the sample beam of the flow cytometer.

The instrument setup was controlled daily with fluorescent reference beads (FCSC, Research Triangle Park, Raleigh-Durham, NC). Monitoring of instrument setup for intensity and color compensation was threefold: (i) using lymphocytes of normal persons according to the AUTOcomp software (Becton-Dickinson); (ii) CD4-FITC (SK3; Becton-Dickinson)/CD8-PE (SK1; Becton-Dickinson)/CD3-PerCP(SK7; Becton-Dickinson) triple staining of peripheral blood from normal donors selected for erythrocyte or platelet transfusions; and (iii) standardized fluorescent beads (FluoroSpheres, DAKO).

Fluorescence was collected at 512–547 nm, 572–591 nm, and >610 nm in the FITC, PE, and PerCP/PE-Cy5 fluorescence light channels. Fluorescence compensation was adjusted by hardware circuits. The amplification for FSC and SSC signals was linear, while fluorescence signals were amplified by four decade logarithmic amplifiers. All data were collected in list mode and transferred to a MS-DOS PC via electronic network for automated classification.

List Mode Analysis

List mode analysis and result classification were performed with the CLASSIF1 program system (Partec, Münster, Germany). Lymphocyte, monocyte, and granulocyte gates in the FSC/SSC histogram were automatically set. The logically leading rectangular lymphocyte gate started with predefined border distances to the left and right, as well as to the upper and lower side of the lymphocyte peak (Fig. 1A,B). The monocyte gate touched the lymphocyte gate with a total extension on the SSC ordinate of 1.5 times the lymphocyte gate. The granulocyte gate touched the monocyte gate and extended until the high end of the SSC ordinate. The left and right border of the monocyte and granulocyte clusters were set in predefined distances to the respective peaks. The preset autogating function was capable of correctly gating all list mode files (about 2,100 files) of the learning and test set without human interference. The outreach of the left and right borders of the evaluation windows was such that on average more than 95% of all leukocytes were enclosed in the three light-scatter evaluation windows.

The two separation lines determining the quadrants in the FITC/PE, FITC/PerCP, or FITC/PE-Cy5 and the PE/

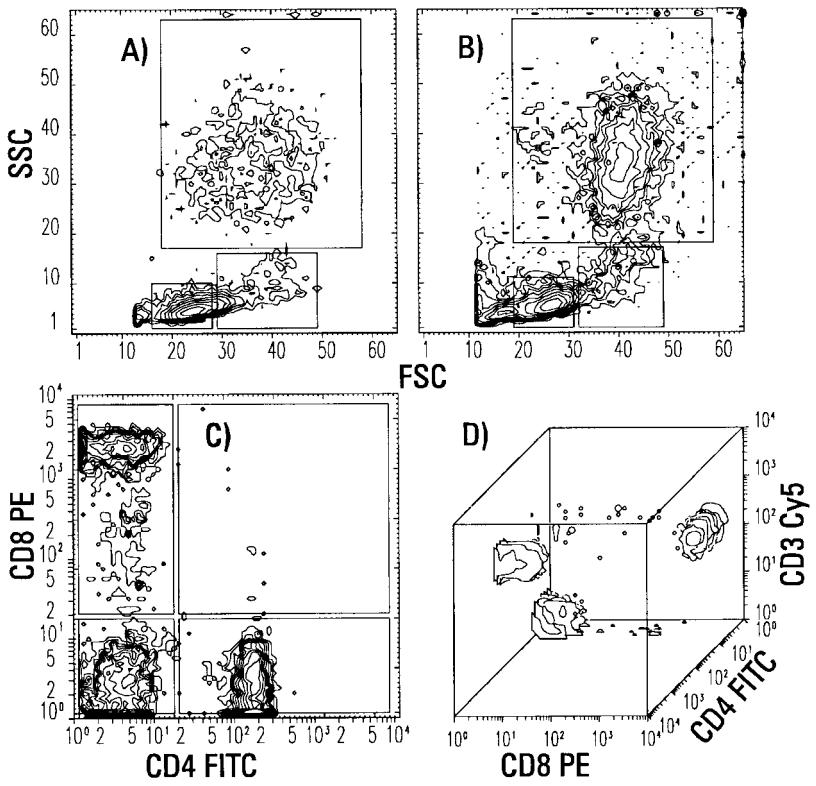


FIG. 1. Automated FSC/SSC lymphocyte, monocyte, and granulocyte gating of two different peripheral blood leukocyte samples **(A,B)**. The fluorescent antibody quadrant analysis was performed with fixed boundaries at 1/3 of the respective fluorescence scales **(C)**. The simultaneous display of the three fluorescences for lymphocytes **(D)**, monocytes (not shown), or granulocytes (not shown) permitted a quick qualitative evaluation of antigenic pattern changes.

PerCP or PE/PE-Cy5 histograms were fixed for all evaluations at one-third of the fluorescence scale in abscissa and ordinate direction (Fig. 1C). Fluorescence histograms were evaluated for the FSC/SSC gated lymphocytes, monocytes, and granulocytes separately as 3D cubes (Fig. 1D) and two parameter histograms (Fig. 1C); i.e., the results of nine two-parameter fluorescence histograms per measurement were available. 74 parameters were extracted for the lymphocyte (Table 1) and similarly for the monocyte and granulocyte cell populations, i.e., 222 parameters for the joint evaluation of all three cell populations per one three-color measurement. A total of $5 \times 222 = 1{,}110$ database columns were extracted for each patient with 5 antibody triplet measurements.

Data Classification

The histological examination of lymph node specimens of the patients of the learning set according to the Kiel classification served as clinical diagnosis (truth) for the subsequent learning process. The learning process proceeds in short as follows:

The classif1 program determines percentile pairs, e.g., 10% and 90% percentiles for the value distribution of the reference samples of the first database column (Fig. 2A). After this, all values of this database column are transformed into triple matrix characters by assigning: 0 to values between the percentiles, + to values above the upper, and − to values below the lower percentile (Fig. 2B). The same is done for all other columns of the learning set database. A triple matrix replica of the numeric database is available after this data transformation step.

The confusion matrix between the known clinical diagnosis of the patients of the learning set on the ordinate and the computer classification of the cytometrically determined immunological database parameters on the abszissa (e.g., Fig. 3) is optimized during the subsequent learning process, using the triple matrix database. The values in each diagonal box of the confusion matrix are ideally 100% when all patient samples are correctly classified (e.g., Fig. 3B). This is not the case in the beginning of the iterative optimization procedure. The CLASSIF1 program successively eliminates all database columns, i.e., cell parameters that neither alone nor in a maximally threefold combination with other database parameters improve the sum of the diagonal values. Once no significant improvement is reached any more ($<0.1\%$ per iteration cycle), the optimization process stops.

Following the learning process, each patient sample of the learning set is reclassified according to the highest positional coincidence of its triple matrix (e.g., Fig. 4, lower box, right) with one of the reference classification masks (e.g., Fig. 4, upper box, right). The reference classification masks contain the most frequent triple matrix character for each database column and patient group

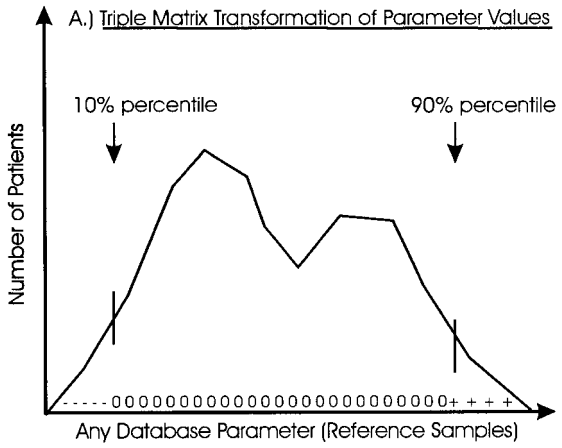
Table 1
Three-Color Immunophenotype List Mode Data Analysis: Calculated Parameters for Lymphocytes

1 = TOTAL CELLS	28 = FITC Ab FITC/PE POS/NEG
2 = % LYMPHOCYTES	29 = PE Ab FITC/PE POS/NEG
	30 = PE/FITC RATIO FITC/PE POS/NEG
FITC Neg/Pos lymphocytes	31 = % FITC/PE POS/POS
3 = % FITC NEG	32 = FITC Ab FITC/PE POS/POS
4 = FSC FITC NEG	33 = PE Ab FITC/PE POS/POS
5 = Ab FITC NEG	34 = PE/FITC RATIO FITC/PE POS/POS
6 = Ab SRF.DENS.FITC NEG	
7 = % FITC POS	PE/CY5 quadrant evaluation
8 = FSC FITC POS	43 = % PE/CY5 NEG/NEG
9 = Ab FITC POS	44 = PE Ab PE/CY5 NEG/NEG
10 = Ab SRF.DENS.FITC POS	45 = CY5-Ab PE/CY5 NEG/NEG
	46 = CY5/PE RATIO PE/CY5 NEG/NEG
PE Neg/Pos lymphocytes	47 = % PE/CY5 NEG/POS
11 = % PE NEG	48 = PE Ab PE/CY5 NEG/POS
12 = FSC PE NEG	49 = CY5 Ab PE/CY5 NEG/POS
13 = Ab PE NEG	50 = CY5/PE RATIO PE/CY5 NEG/POS
14 = Ab SRF.DENS.PE NEG	51 = % PE/CY5 POS/NEG
15 = % PE POS	52 = PE Ab PE/CY5 POS/NEG
16 = FSC PE POS	53 = CY5 Ab PE/CY5 POS/NEG
17 = Ab PE POS	54 = CY5/PE RATIO PE/CY5 POS/NEG
18 = Ab SRF.PE POS	55 = % PE/CY5 POS/POS
	56 = PE Ab PE/CY5 POS/POS
CY5 Neg/Pos lymphocytes	57 = CY5 Ab PE/CY5 POS/POS
35 = % CY5 Ab NEG	58 = CY5/PE RATIO PE/CY5 POS/POS
36 = FSC CY5 Ab NEG	
37 = CY5 Ab NEG	FITC/CY5 quadrant evaluation
38 = CY5 Ab SURG DENS NEG	59 = % FITC/CY5 NEG/NEG
39 = % CY5 Ab POS	60 = FITC Ab FITC/CY5 NEG/NEG
40 = FSC CY5 Ab POS	61 = CY5 Ab FITC/CY5 NEG/NEG
41 = CY5 Ab POS	62 = CY5/FITC RATIO FITC/CY5 NEG/NEG
42 = CY5 Ab SURG DENS POS	63 = % FITC/CY5 NEG/POS
	64 = FITC Ab FITC/CY5 NEG/POS
FITC/PE quadrant evaluation	65 = CY5 Ab FITC/CY5 NEG/POS
19 = % FITC/PE NEG/NEG	66 = CY5/FITC RATIO FITC/CY5 NEG/POS
20 = FITC Ab FITC/PE NEG/NEG	67 = % FITC/CY5 POS/NEG
21 = PE Ab FITC/PE NEG/NEG	68 = FITC Ab FITC/CY5 POS/NEG
22 = PE/FITC RATIO FITC/PE NEG/NEG	69 = CY5 Ab FITC/CY5 POS/NEG
23 = % FITC/PE NEG/POS	70 = CY5/FITC RATIO FITC/CY5 POS/NEG
24 = FITC Ab FITC/PE NEG/POS	71 = % FITC/CY5 POS/POS
25 = PE Ab FITC/PE NEG/POS	72 = FITC Ab FITC/CY5 POS/POS
26 = PE/FITC RATIO FITC/PE NEG/POS	73 = CY5 Ab FITC/CY5 POS/POS
27 = % FITC/PE POS/NEG	74 = CY5/FITC RATIO FITC/CY5 POS/POS

of the learning set. A total or partial (e.g., Table 2) parameter list of the reference classification masks facilitates the understanding of the optimized parameter pattern. The reclassification of the learning set samples permits a visual check of patient triple matrices for systematic deviations (e.g., with time) from the reference classification masks (Fig. 4).

The quality of the overall classification is judged in a standardized way by the average recognition index (ARI) and average multiplicity index (AMI) parameters. The ARI represents the sum of the diagonal values of the confusion matrix divided by the number of classification states. It should be higher than 80% for clinical purposes. The AMI is a measure for the assignment of more than one classification state to a sample. The AMI is ideally 1.0 in the absence of multiple classifications and 1.1, 1.2, 1.33 when every tenth, fifth, or third example on average is assigned a double classification. AMIs of 1.0–1.2 are acceptable in practice. The AMI is calculated as ratio between the sum of all line sums of the confusion matrix divided by the number of classification states and then by 100.

The classifier learning time with the maxium dataset of 65 patients containing 1,100 database columns was around 15 min on a 90-MHz Pentium PC. The classification time per patient for the five three-color assays was less than 2 min. This is faster than the measurement time of the five



B.) Database Conversion of Parameter Values

	numeric database parameters			triple matrix database parameters		
patient	1	2	3	1	2	3
1	40.1	4.02	18.38	0	+	0
2	39.5	4.20	5.25	-	+	-
3	41.2	3.46	35.35	0	-	+
4	53.2	3.78	30.72	+	0	+
5	41.3	3.95	27.46	0	0	0
6	79.3	3.80	16.29	+	0	0
7	48.0	3.98	28.33	0	0	0
⋮	⋮	⋮	⋮	⋮	⋮	⋮

FIG. 2. Schematic representation of the value distribution of a single database column for all reference patients **(A)**. Two percentiles (e.g., 10% and 90%) are calculated for this distribution. All values of the database column; i.e., the values of the reference and diseased patients are subsequently transformed into triple matrix characters according to their position towards the two percentile thresholds, i.e., below the lower, 0 between both, and + above the upper percentile (**B**). The triple matrix database is classified in a standardized way; i.e, all database columns are eliminated that deteriorate the classification result, either alone or in combination with other database columns.

cell assays; i.e., classification can be performed on-line. More than 90% of the classification time is consumed by list mode evaluation and plot file generation.

RESULTS

The first classification goal was to discriminate between normal persons, LP-IC and B-CLL patients based on blood (Fig. 3A) and bone marrow (Fig. 3B) samples by using the information of all three cell populations (LMG) and all five antibody triplets. Both classifications identify the normal and abnormal samples correctly (100.0%) at a low multiplicity index (AMI) of 1.02 and 1.00, i.e., with high certainty.

The previous and all following classifications were optimized by calculating the classifications for the 5/95%, 10/90%, 15/85%, 20/80%, 25/75%, and 30/70% percentile pairs. The best classifications were usually obtained between the 10%/90% and 25%/75% percentile pairs with an optimum at the 15%/85% or 20%/80% setting.

The triple matrix pattern (Fig. 4) of the reclassified learning set (Fig. 3A) shows the classification masks of the different samples. Most of the samples are correctly classified although none of the samples coincides fully (1.00 in column #4 of Fig. 4) with the reference classification masks. This demonstrates the inherent error tolerance of the triple matrix classification method.

Out of the initial 1,110 database columns, only 28 (Table 2) and 38 (Table 3) columns, i.e., 2.5% and 3.4%, were informative for the clinical patient status and included into the reference classification masks. Seventeen and 23 of the selected columns served for the discrimination between normal and malignant (not shown), while 11 (Table 2) and 15 (Table 3) columns contained the discriminant information between LP-IC and B-CLL patients. These columns concern preferentially monocyte and granulocyte parameters.

In a second classification, the contribution of each antibody triplet alone was evaluated for all cells (LMG). The classification results for blood (Fig. 5A) and bone marrow (Fig. 5B) were similar to the results obtained with all five antibody triplets in case of the lambda/CD19/5 antibody triplet but more classification parameters 42 (15 lymphocyte, 13 monocyte, 14 granulocyte) and 47 (16 lymphocyte, 13 monocyte, 18 granulocyte) were selected. This shows that several of the antibody triplets contain redundant information. Thirteen and 19 parameter differentiated between LP-IC and B-CLL. A substantial part of the differential parameters concerned lymphocyte parameters (5 and 7).

The two classification approaches show that individual cell populations contributed different amounts of information to the combined LMG analysis with five antibody triplets (Table 4A). While lymphocyte results alone provided 82.8% and 93.3% correct classifications, the inclusion of the monocyte and granulocyte information increased the correct classification to 100.0% for peripheral blood or bone marrow samples (Fig. 3A,B); i.e. nonlymphoid cells contributed significant information to the optimal classification result. The results show also that the restriction to monocyte or granulocyte parameters, still provides a significant degree of correct classifications, i.e., 88.1–95.2%.

The results indicate furthermore that, e.g., the restriction of the information to the single lambda/CD19/5 antibody triplet for LMG analysis (Table 5A) provided 95.2% and 100.0% correct classifications for blood and bone marrow cells (Fig. 5A,B). This is almost as good as the LMG analysis results with five triplets (Table 4A). The remaining other triplets permit correct classification at somewhat lower levels between 84.1% and 100.0% of the cases. This demonstrates in greater detail the redundancy of information obtained from the different antibody triplets.

In the third classification task, unspecified leukocytosis cases were included into the learning set. The average classification for peripheral blood leukocyte samples decreased to 89.3% for the LMG analysis with all antibody triplets (Fig. 6A) and to 86.0% for the LMG analysis with

CONFUSION MATRIX (% PAT)
DATABASE: OFLEARN.BI4

CLIN. DIAG.	PAT. n	FLOW-CLASSIF. PN	PL	PY
PN	15	100.0	.0	.0
PL	14	.0	100.0	7.1
PY	9	.0	.0	100.0

PN =PBLNOR
PL =PBLCLL
PY =PBLICY
aver.recog.ind(ARI): 100.0%
aver.multipl.ind(AMI): 1.02
(acceptable range: 1.0-1.2)
differential classification
percentile range: 20-80%

A

CONFUSION MATRIX (% PAT)
DATABASE: OPLEARN.BI4

CLIN. DIAG.	PAT. n	FLOW-CLASSIF. KN	KL	KY
KN	15	100.0	.0	.0
KL	7	.0	100.0	.0
KY	5	.0	.0	100.0

KN =BMNOR
KL =BMCLL
KY =BMICY
aver.recog.ind(ARI): 100.0%
aver.multipl.ind(AMI): 1.00
(acceptable range: 1.0-1.2)
differential classification
percentile range: 10-90%

B

FIG. 3. Confusion matrices for the classification of peripheral blood leukocyte **(A)** and bone marrow **(B)** samples of normal (PBLNOR, BMNOR) persons, as well as B-CLL (PBLCLL, BMCLL) and LP-IC (PBLICY, BMICY) patients. Optimal classification results were obtained with 20/80% and 10/90% percentiles using the differential classification mode. Lymphocyte, monocyte, and granulocyte information from the CD4/8/3, kappa/CD19/5, lambda/CD19/5, CD45/14/20, CD10/23/19 measurements were included in the classification database. The above results were obtained with only 28 (Table 2) and 38 (Table 3) out of the original 1,110 database columns. The remaining columns did neither alone nor in combination improve the classification result.

the single lambda/CD19/5 antibody triplet (Fig. 6B); i.e., some loss of specificity occurred.

The simultaneous classification of normals, patients with unspecified leukocytosis, LP-IC and B-CLL together with 6 additional types of lymphomas in the fourth classification task did not further lower the specificity of the classification (Fig. 7), since 88.9% of the samples were correctly classified at the low average multiplicity index (AMI) of 1.01, i.e., with high certainty. Lymphocyte parameters were quite important in this classification (88.5%, 88.6%, Table 4B), with an only small increment to 92.2% and 89.9% upon inclusion of monocyte and granulocyte data. The single CD4/8/3 antibody triplet was most successful for peripheral blood leukocytes (86.4%, Table 5B), while the kappa/CD19/5 triplet best identified bone marrow samples (90.0%). Even this example with the high number of different classification categories shows that the number of antibody measurements can be substantially reduced with optimized antibody combinations.

The fifth classification consisted in the identification of unknown samples of the two test sets. The unknown normal/LP-IC/B-CLL blood samples (Table 6) were classified with very similar results as the samples of the learning set (Fig. 3A); i.e., the classifier is robust toward unknown samples. For the second test set of unclassifiable lymphoma patients or patients suspicious of lymphoma, the CLASSIF1 program classified 19 out of 20 cases and gave one ambiguous (LP-IC/B-CLL) judgment. Although imprecise, the judgment still puts the sample onto the pathological side; i.e., the patient is flagged as abnormal.

DISCUSSION

The requirement for automated classification of multiparametric flow and image cytometry data constitutes an increasingly important issue. Since diseases originate from biochemical changes in cellular systems or organs, the multiparametric approach of cytometry is conceptually very promising indeed for diagnostic, prognostic and therapy control purposes in hematology and medicine in general.

The traditional computer-assisted manual interpretation of flow cytometric histograms is self-limiting due to the inherent complexity of multiparameter data, the infinite number of possible permutations in antibody combinations, the high data output from multiple multiparameter measurements, and the difficulty to develop generally agreed and clear-cut classification rules. Morphological classification using cell and histomorphological features of lymphoid tissues (2,5,19,21) for the distinction of lymphoma subtypes is therefore still the method of choice in lymphoma classification.

Nevertheless, several strategies for the automated interpretation of flow cytometric multiparameter results have been used. Rule- or knowledge-based systems (8,30) exploit only the restricted expert knowledge on the currently interpretable fraction of the total information con-

NR.	CLINICAL DIAGNOSIS	ABBREVIATION	COIN	REF.CLASSIFIC.MASKS
1	PBLNOR	PN	1.00	00000000000000000000000000
2	PBLCLL	PL	1.00	+--+--++--0-++-++0-+-0+0-000
3	PBLICY	PY	1.00	+--+0-++--+0++-++-++--++0-++

REC. NR.	DATAB: OFLEARN.BI4 DATAB.RECORD LABEL	FLOW-CLASSIFICATION	CLAS COIN	SAMPLE CLASSIF.MASKS . = no value
78	N001P.....N.....	PN	.86	000+000--+0-0-++0+0000000+0-
79	N002P.....N.....	PN	.82	0-++-++000-+00+000000+-0+00+
80	N003P.....N.....	PN	.68	-+0+0--+0000+00+++-++00--+00
81	N005P.....N.....	PN	.86	0000-++00000000++000+0--+000
82	N006P.....N.....	PN	.93	-0+0+00000000000-+0--+00-0-0
83	N007P.....N.....	PN	.79	+0000+0+-00+++00+000++00+0-0
84	N008P.....N.....	PN	.68	0+-0---+-+-+-0++0++000+0+0+0
85	N009P.....N.....	PN	.93	00+-+000++0000+0000+-0000+00
86	N010P.....N.....	PN	.86	+0-000+0+000-000000-0--00000
87	N011P.....N.....	PN	.64	+00+00+-0-000+--00-0+0+-0-0+
88	N012P.....N.....	PN	.71	00+0000++-+0000000+00-00---+
89	N013P.....N.....	PN	.82	00000+00+00-0+0-0-00000+00+0
90	N014P.....N.....	PN	.75	+-00+-000-+-0-00-00-0-000+0-
91	N015P.....N.....	PN	.68	0-0-000000+0+0-00-+000++00+0
92	N016P.....N.....	PN	.79	0+0-0000+000-+0-+00+000+00++
16	2208P.....L.....	PL	.75	+--+--+00-0-+0-000-+-0+000-0
20	2234P.....L.....	PL	.82	+--+--++--00++-+0--+00+00000
53	2072P.....L.....	PL	.71	+--++-++--+0++-++000-++0---0
57	2096P.....L.....	PL	.75	+--+--+0--+-+--++0++-++0-+-0
60	2125P.....L.....	PL,PY	.79	+--+--++--00++-++0++-0++0++0
64	2164P.....L.....	PL	.71	+--+--++--000+-++-00--+0000-
111	2361P.....L.....	PL	.75	+--+--++--+-++-+++-0-----00-
117	2289P.....L.....	PL	.71	+--+--++-000++-+-+0+0++0-0-0
119	2323P.....L.....	PL	.82	+--+--++--0-+0-+00-0-0---000
120	2324P.....L.....	PL	.64	+--+--+-0+--++00+000-0+0000-
122	2329P.....L.....	PL	.75	+--+--++0-0-++-0+--+-0+-0--0
123	2348P.....L.....	PL	.71	+--+--+0--0-0--+00-0--+--00-
125	2352P.....L.....	PL	.82	+--+--++--00++-++0-0--+0000-
126	2353P.....L.....	PL	.82	+--+--++--0-++-++-0+--+0-0--
9	2064P.....Y.....	PY	.61	0+-+0-0+--+0++-++0+-00000-00
13	2078P.....Y.....	PY	.57	0-+-000+--+0++-++00++-00+0++
14	2163P.....Y.....	PY	.89	+--+--++--+0++-++-+0--++0-+0
15	2199P.....Y.....	PY	.82	+--00-++--00++-+0-+0+-++0-++
54	2077P.....Y.....	PY	.82	+--+--++--+0++-++-++-++++---
55	2082P.....Y.....	PY	.75	+--00-++--+0++-++00+-++00-0-
61	2135P.....Y.....	PY	.57	++0000++--000+00+-+++++0-00
121	2327P.....Y.....	PY	.82	+--0+-++---0++-++-++0-+00-++
124	2349P.....Y.....	PY	.89	+--+--++--+0++-++--+--+++-++

FIG. 4. Reclassification of the learning set for the peripheral blood leukocyte samples of Fig. 3A against the three reference classification masks (top of table column #5). The reclassification shows that the clinical diagnosis (N = normal, L = B-CLL, Y = LP-IC) in the second column is well recognized by the flow classifier (PN = normal, PL = B-CLL, PY = LP-IC) in the third column. The triple matrix pattern of the reclassified samples (fifth column) shows no systematic deviations with time (increasing patient numbers). The patterns also demonstrate the inherent error tolerance (fourth column) of the classification algorithm. Most samples are correctly classified although none of the samples (0.57-0.93) shows complete coincidence 1.0 with the reference classification masks. The reference classification masks contain the most frequent triple matrix character of each data column for the PN, PL, and PY patient groups. Samples are reclassified according to the highest positional coincidence with any one of the classification masks. Multiple classifications (e.g., patient #2125 PL, PY) are provided at equal coincidence frequencies for more than one disease state.

Table 2
B-CLL/LP-IC Differential Classification: Parameters for Peripheral Blood Leukocytes[a]

Parameter of ref class mask	Cell type	Antibody triplet	Two-parameter histogram used for parameter extraction	Ab expression in two-parameter histogram quadrant	Ref class mask B-CLL	Ref class mask LP-IC
5	lym	CD45/14/20	CD45/20	CD20/45 Ab ratio, pos/pos	−	0
12	mon	CD45/14/20	CD14/20	CD20 Ab, neg/pos	−	0
11	mon	κ/CD19/5	κ/CD19	κ Ab, pos/pos	0	+
23	gran	CD45/14/20	CD14/20	CD14 Ab, pos/pos	0	+
18	gran	CD4/8/3	CD4/8	CD4 Ab neg/pos	0	−
19	gran	CD4/8/3	CD4/8	CD8/4 Ab ratio, pos/pos	−	+
25	gran	CD4/8/3	CD8/3	CD3/4 Ab ratio, neg/pos	−	0
26	gran	CD4/8/3	CD8/3	CD3/4 Ab ratio, pos/neg	0	−
22	gran	CD10/23/19	CD10/23	CD10 Ab, pos/pos	0	−
27	gran	CD10/23/19	CD23/19	CD19 Ab, neg/pos	0	+
28	gran	CD10/23/19	CD23/19	CD19/23 Ab ratio, neg/pos	0	+

[a]Total of 28 classification parameters (n = 7 lymphocytes, n = 10 monocytes, n = 11 granulocytes). Displayed are the 11 parameters discriminating between B-CLL and LP-IC. The remaining 17 parametes discriminate between the normal and malignant immunophenotype. No percentage cell frequency value is selected. The triple matrix characters in the last two columns represent the discriminating parameters selected from the reference classification masks of Fig. 4 (top of the rightmost column).

Table 3
B-CLL/LP-IC Differential Classification: Parameters for Bone Marrow Cells[a]

Parameter of ref class mask	Cell type	Antibody triplet	Two-parameter histogram used for parameter extraction	Ab expression or % cell frequency in two-parameter histogram quadrant	Class mask B-CLL	Class mask LP-IC
7	lym	κ/CD19/5	κ/CD19	CD19 Ab, pos/neg	+	0
13	mon	CD45/14/20	CD45/14	CD45 Ab, neg/pos	+	0
14	mon	CD45/14/20	CD45/14	CD14 Ab, neg/pos	+	0
15	mon	CD45/14/20	CD45/14	CD14/45 Ab ratio, neg/pos	+	0
17	mon	κ/CD19/5	κ/CD19	CD19 Ab, neg/neg	0	+
21	mon	κ/CD19/5	CD19/5	CD19 Ab, neg/neg	0	−
22	mon	λ/CD19/5	CD19/5	CD19 Ab, neg/neg	0	−
23	mon	λ/CD19/5	CD19/5	CD5/19 Ab ratio, neg/neg	0	+
24	mon	λ/CD19/5	CD19/5	% CD19/5, pos/neg	+	−
16	mon	CD4/8/3	CD4/8	CD8 Ab surf dens. neg	0	−
27	gran	CD45/14/20	FSC/SSC	% granulocytes	−	0
28	gran	κ/CD19/5	κ/CD19	CD19 Ab pos	-	−
31	gran	κ/CD19/5	CD19/5	% CD19/5, pos/neg	0	−
35	gran	λ/CD19/5	CD19/5	% CD19/5, pos/neg	0	−
38	gran	CD10/23/19	CD23/19	CD19/23 Ab ratio, neg/pos	−	+

[a]Total of 38 classification parameters (n = 12 lymphocytes, n = 14 monocytes, n = 12 granulocytes). Displayed are the 15 parameters discriminating between B-CLL and LP-IC. The remaining 23 parameters discriminate between the normal and malignant immunophenotype. Only 4 of 15 parameters represent percentage cell frequency values. The triple matrix characters in the last two columns represent the discriminating parameters, selected from the reference classification masks of the bone marrow samples (not shown).

tent of multiparameter cytometric measurements. Problems in cluster analysis (7,24,33) concern the meaningful definition of biologically relevant clusters in the learning data set and the handling of lost or new clusters in pathological conditions. Neural networks in cytometry classify certain cell types quite well (2,3,5) but cannot separate others due to insufficient specific information from single list mode measurements. They also require long training times for multiple multiparameter measurements. Statistical classifiers (23) may produce unstable results, especially when few informative parameters are embedded in a multitude of noninformative parameters, as in multiparameter cytometry. Hierarchical classifiers (1,6,30) are difficult to adapt to the complexity of the hematopoietic system, while the understanding of the classification parameters is frequently difficult in multivariate discriminant analysis, fuzzy logic (20) and in principal component analysis (18). Further problems concern the fast adaptation to new tasks, the generation of nonintuitive classification parameters, the dependence of the classification process from interlaboratory accuracy and the lack of standardized, i.e., portable classifiers.

The triple matrix pattern classification algorithm (12,28,31,32), in contrast, provides robust and standardized classifiers. The selected classification parameters are understandable because they consist of unmodified database parameters. No prior knowledge on the data distribution is necessary and there are no underlying assumptions in the classification process. Learning is fast because computer operations consist only in comparisons of indi-

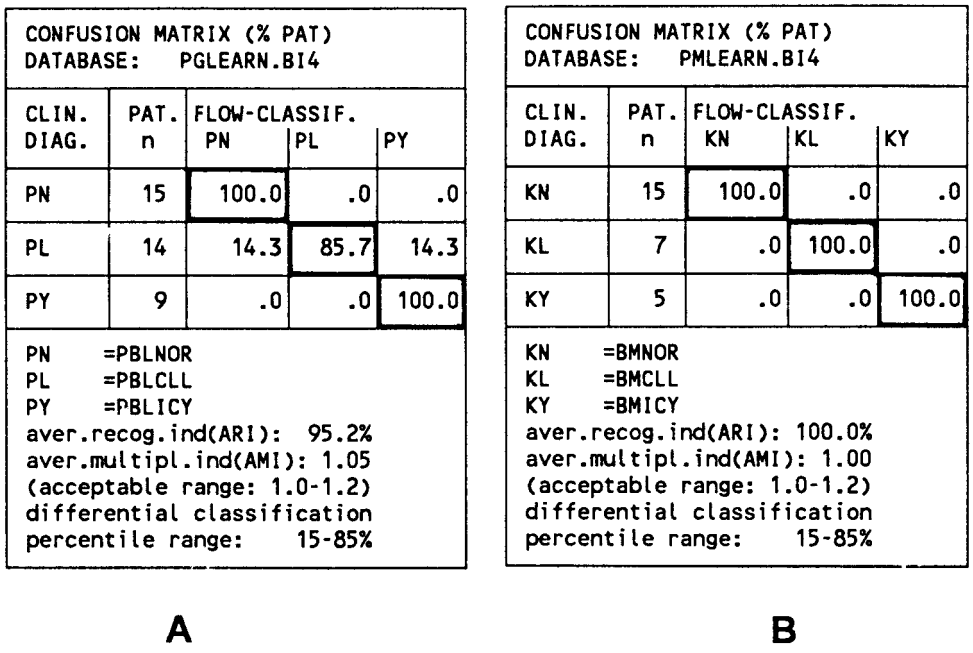


FIG. 5. Similar classification results for peripheral blood leukocyte **(A)** and bone marrow samples **(B)** as in Fig. 3 were obtained when only the lambda/CD19/5 database was jointly evaluated for lymphcytes, monocytes, and granulocytes. This indicates that the five antibody triplet immunophenotype pattern for Fig. 3 was substantially overdetermined with regard to the distinction between normal, B-CLL and LP-IC samples. Only 42 and 47 database columns (18.9%, 21.1%) of the 222 columns of the entire database were used for the classification, while the remaining columns were not informative. Abbreviations as in Fig. 3.

Table 4
Classification for Lymphocytes, Monocytes, and Granulocytes

Sample	%	Lymphocytes	Monocytes	Granulocytes	Lymphocytes + granulocytes	Lymphocytes + monocytes + granulocytes
			A. B-CLL/LP-IC/Normal Samples (ARI %/AMI)[a]			
PBL	20–80	82.8/1.09	88.1/1.17	88.0/1.07	88.9/1.00	100.0/1.02
BM	10–90	93.3/1.00	95.2/1.00	93.0/1.00	100.0/1.00	100.0/1.00
			B. High- and Low-Grade Lymphomas, B-CLL, Leukocytosis, and Normal Cell Samples[b]			
PBL	15–85	88.5/1.03	85.8/1.03	80.7/1.09	88.9/1.01	92.2/1.08
BM	20–80	88.6/1.01	79.7/1.05	88.9/1.03	85.7/1.01	89.9/1.08

[a]Percentiles (%), number of patients, as in Figs. 3A, B and 5A, B.
[b]Normal (n = 15), CLL (14), hairy cell leukemia (8), LP-IC (9), centroblastic (2), centrocytic-centroblastic (2), B-cell lymphoma (1), centrocytic (1), low-grade lymphoma (1), unspecific leukocytosis (12).

vidual parameters with the upper and lower percentile thresholds of the reference parameter distributions. The classifiers rely on representative and well-characterized learning sets, on intralaboratory measurement precision and on quality reagents for antigen staining. In this respect, the triple matrix classifier requirements are similar to other classification methodologies.

The three learning sets of this study were correctly classified with single patient recognition frequencies of 85.7–100.0% in the three-category (Figs. 3, 5) classification, 75.0–100.0% in the four-category (Fig. 6) classification, and 55.6–100.0% in the 10-category (Fig. 7) classification. The classifiers were robust for unknown test samples (Table 6), and clear classification results were provided in 18 out of 19 otherwise unclassifiable blood as well as bone marrow samples (Table 7).

The analysis of the selected classification parameters shows that only few parameters, i.e., 2.5% (Table 2) of the available parameters for blood leukocytes and 3.4% (Table 3) for bone marrow cells, are relevant for the reference classification masks while most parameters are rejected during the optimization process. This parallels earlier classifications with nonmalignant diseases (31) and emphasizes the point that the success oriented selection in combination with the ultimate elimination of less successful parameters is very essential for the entire classification process because the ''noise'' level of unrelated parameters is substantially lowered in this way.

The reduction of the classification parameters to the most informative parameters avoids the problem of statistically overdetermined learning sets. The number of 1,110- or 222-database columns as classification parameters in the

Table 5
Classification by Immunophenotypes

Sample	%	CD45/14/20	CD4/8/3	κ/CD19/5	λ/CD19/5	CD10/23/19	All antib.
A. B-CLL/LP-IC/Normal Samples (ARI %/AMI)[a]							
PBL	15–85	89.2/1.05	84.1/1.02	87.8/1.02	95.2/1.05	94.1/1.02	91.7/1.00
BM	15–85	93.3/1.00	93.3/1.05	100.0/1.00	100.0/1.00	95.2/1.00	93.3/1.00
B. High and Low Grade Lymphomas, B-CLL, Leukocytosis, and Normal Cell Samples[b]							
PBL	25–75	83.1/1.05	86.4/1.07	83.9/1.06	86.0/1.08	80.4/1.09	71.4/1.02
BM	20–80	88.6/1.03	80.3/1.06	90.0/1.00	82.2/1.03	89.9/1.06	82.7/1.01

For explanations, see Table 4.

CONFUSION MATRIX (% PAT)
DATABASE: QRLEARN.BI4

CLIN. DIAG.	PAT. n	FLOW-CLASSIF. PN	PL	PY	PZ
PN	15	93.3	6.7	.0	.0
PL	14	.0	100.0	.0	.0
PY	9	.0	.0	88.9	22.2
PZ	12	8.3	.0	16.7	75.0

PN =PBLNOR
PL =PBLCLL
PY =PBLICY
PZ =PBLCYT
aver.recog.ind(ARI): 89.3%
aver.multipl.ind(AMI): 1.03
(acceptable range: 1.0-1.2)
differential classification
percentile range: 25-75%

A

CONFUSION MATRIX (% PAT)
DATABASE: QOLEARN.BI4

CLIN. DIAG.	PAT. n	FLOW-CLASSIF. PN	PL	PY	PZ
PN	15	100.0	.0	.0	.0
PL	14	7.1	85.7	28.6	.0
PY	9	.0	22.2	66.7	22.2
PZ	12	8.3	.0	.0	91.7

PN =PBLNOR
PL =PBLCLL
PY =PBLICY
PZ =PBLCYT
aver.recog.ind(ARI): 86.0%
aver.multipl.ind(AMI): 1.08
(acceptable range: 1.0-1.2)
differential classification
percentile range: 25-75%

B

FIG. 6. The addition of samples from unspecified leukocytosis patients (PBLCYT) to the blood cell classification database containing all cells (LMG) and all five antibody triplets **(A)** indicates that the leukocytosis samples are confused to some extent with samples from normal persons and also with LP-IC immunocytoma samples. The identification decreases slightly from 89.3% to 86.0% when only the lambda/CD19/5 data from all three cell types is used for classification **(B)**. Like before the results are obtained with comparatively few informative database columns (35/1,110 (3.1%), 40/222 (18.0%))

initial dataset is substantially higher than the number of patients in the blood leukocyte (n = 36) and bone marrow (n = 27) learning set, but the number of finally selected parameters is only 28–47, i.e., either lower or in the same order as the number of patients.

It is surprising that lymphocyte parameters constitute a relative minority in the final reference classification masks, e.g., 25% (Table 2, Fig. 3A) for blood and 31.5% (Table 3, Fig. 3B) for bone marrow samples and that comparatively few percentage frequency values of cell populations are selected, while total amount of cellular antigen, relative antigen density, and antigen ratios occur much more frequently (Tables 2, 3). This shows that the discrimination efficiency for various lymphoma types depends less on the appearance or disappearance of particular cell populations but rather on the quantitative makeup of leukemic and lymphoma cell populations and on the joint evaluation of parameters from lymphocytes, monocytes, and granulocytes (Table 4A). This may be the consequence of altered differentiation patterns at the stem cell level, as well as the consequence of functional interactions between normal and malignant cells at later stages of differentiation.

The extension of the classification categories to 10 classification states (Table 4B, Fig. 7), including normal and unspecified leukocytosis patients, provided best results for the lymphocyte classification alone. In this case, the lymphocytes carry the highest information for the fine tuned differentiation between various low- and high-grade lymphomas.

CONFUSION MATRIX (% PAT)
DATABASE: RCLEARN.BI4

CLIN. DIAG.	PAT. n	FLOW-CLASSIF. PN	PZ	PL	PC	PE	PB	PA	PY	PD	PG
PN	15	93.3	.0	.0	.0	.0	.0	6.7	.0	.0	.0
PZ	12	.0	66.7	16.7	.0	.0	8.3	.0	8.3	.0	.0
PL	14	.0	.0	85.7	.0	7.1	.0	7.1	.0	.0	.0
PC	8	.0	.0	.0	87.5	.0	.0	.0	12.5	.0	.0
PE	1	.0	.0	.0	.0	100.0	.0	.0	.0	.0	.0
PB	2	.0	.0	.0	.0	.0	100.0	.0	.0	.0	.0
PA	2	.0	.0	.0	.0	.0	.0	100.0	.0	.0	.0
PY	9	.0	22.2	22.2	.0	.0	.0	11.1	55.6	.0	.0
PD	1	.0	.0	.0	.0	.0	.0	.0	.0	100.0	.0
PG	1	.0	.0	.0	.0	.0	.0	.0	.0	.0	100.0

PN =PBLNOR
PZ =PBLCYT
PL =PBLCLL
PC =PBLHCL
PE =PBLCCL
PB =PBLCB-CCL
PA =PBLCBL
PY =PBLICY
PD =PBLBCL
PG =PBLLGR
aver.recog.ind(ARI): 88.9%
aver.multipl.ind(AMI): 1.01
(acceptable range: 1.0-1.2)
differential classification
percentile range: 15-85%

FIG. 7. Classification of peripheral blood leukocyte samples from normal persons (PBLNOR), unspecified leukocytosis (PBLCYT), B-CLL (PBLCLL), hairy cell leukemia (PBLHCL), centrocytic (PBLCCL), centrocytic-centroblastic (PBLCB-CCL), centroblastic lymphoma (PBLCBL), LP-IC immunocytoma (PBLICY), B-cell lymphoma (PBLBCL), low-grade lymphoma (PBLLGR), using the lymphocyte and granulocyte information from all five antibody triplet measurements. A total of 46 of the 1,110 database columns (4.1%) were selected for the classification.

Table 6
Classification of Unknown Test Samples

Clinical diagnosis	n	CLASSIF1 classification (%) NORMAL	B-CLL	LP-IC
Normal	11	100.0	0.0	0.0
B-CLL	4	0.0	100.0	25.0
LP-IC	2	0.0	0.0	100.0

Table 7
Classification of Manually Unclassifiable Test Samples

CLASSIF1 classification	Cell type PBL	BM
Normal	2	4
LP-IC	0	3
B-CLL	5	1
Other lymphomas	3	0
Double classification	0	1
Total patients	10	9

The detailed LMG cell analysis for individual antibody triplets reveals the unexpected fact that the individual triplets alone may have quite significant classification capacities with 84.1–100.0% correct results in the B-CLL/LP-IC/Normal classification task. The lambda/CD19/5 (100.0%) was the most discriminant single antibody triplet (Table 5A). Analysis of the 10-category classifier (Fig. 7) demonstrates the same phenomenon (71.4-90.0%) (Table 5B) except that the CD8/4/3 (86.4%) and the kappa/CD19/5 (90.0%) antibody triplet (Table 5B) provide the best individual classification results.

The fact that changes of few antigenic determinants upon simultaneous analysis on lymphocytes, monocytes, and granulocytes (Table 5A, lambda/CD19/5 column) are more discriminant than the analysis of many antigenic determinants on lymphocytes alone (Table 4A, lympho-

cytes against lymphocytes + monocytes + granulocytes column) indicates that the lymphocytic, monocytic, and granulocytic system has to be seen as a vertical, rather than as a horizontal, system of individual cell populations for classification purposes.

The results shown in Table 5 also indicate that the five-triplet antibody panel is substantially overdefined for the discrimination of B-CLL, LP-IC, and normal individuals. The main reason for this is that the panels were derived by cell population oriented, deductive thinking based on the existing knowledge from immunological and clinical lineage development studies. The experience with the cell parameter oriented CLASSIF1 algorithm indicates that the cell population oriented concept (Table 4), although essential for the initial development of classification strategies, provides a multitude of redundant parameters (Table 5). The decisive improvement of automated classification consists in the identification of the minimum efficient antibody panel according to the LMG analysis concept (Table 5).

Similarly, the classification of nonmalignant diseases (12,28,31,32) depends preferentially on parameter quantity and less on the frequency of particular cell populations. Quantitative parameter changes are less perceptible to the human eye in flow cytometric histograms than the appearence or disappearence of discrete cell populations especially in highly compressed four decade logarithmic dataspaces. Therefore, exhaustive parameter classification on all cellular subsets constitutes the important new aspect for automated data classification in blood and bone marrow cytometry.

Data from predefined age and sex matched groups of healthy individuals are used as reference; i.e., all data are expressed as a fraction of the respective mean values of the reference group. This eliminates the influence of systematic interlaboratory accuracy deviations and of slightly different methodology on the classification process. This is important for the development of standardized interlaboratory classifiers. The classifiers rely on intralaboratory precision, which may be within 1–2%, according to ring trial results, while results for interlaboratory accuracy are frequently substantially less consistent.

The use of reference groups instead of absolute cytometrical or biochemical parameter values requires the inter-institution comparability of such groups. The comparability of reference groups is checked by CLASSIF1 cross-classification; e.g., reference groups from different laboratories are classified against consensus reference groups. If no difference is detected, the consensus classifier can be utilized at new locations without new learning on diseased patients groups again. This is an important advantage because it may, in fact, be quite difficult for smaller hospitals or in private practice to obtain sufficient patient numbers to learn, e.g., on rare diseases.

Efforts to explain selected triple matrix parameter patterns, prompt the development of new scientific hypotheses. Since the learning process requires only a few minutes and since most of the parameters are eliminated during the classification process, it is tempting to initially include the totally available cytometric and non cytometric patient parameter information into the disease classification processes in order to provide the highest possible chances for the enrichment of relevant parameters. In line with this idea, the unusual extension of the classification parameters from lymphocytes to monocyte and granulocytes in this study has provided the unexpected finding that monocyte and granulocyte information is important for lymphoma classification and furthermore that the logically conceived five antibody triplets are in practice largely overdetermined.

Future classification work may directly address the correlation between clinical disease progress or response to therapy and immunophenotyping as well as clinical chemistry data in an effort to establish the closest possible link between biochemical parameters and disease development.

The elimination of redundant antibody combinations by standardized classification, may permit the simultaneous classification of most clinically relevant human leukemias and lymphomas from a relatively minor set of optimized three- or four-color cellular immunostains.

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