Technology and New Fluorescence Flow Cytometry Parameters in Hematological Analyzers

Borros M. Arneth* and Mario Menschikowki

Institute of Clinical Chemistry and Laboratory Medicine, Carl Gustav Carus University Hospital, University of Technology Dresden, Dresden, Germany

Background: Modern hematological analyzers offer a series of new tests in addition to the differential blood cell count. Here, we provide a short overview of the technique and the new tests included in the hematological analyzers manufactured by the company Sysmex (Kobe, Japan). As introduced here, the resulting new extended blood cell count allows for the quantification of DNA- and RNA-rich, activated and/or precursor leukocyte subpopulations. **Methods:** To compare the different methods of blood cell counting, repetitive cell count-

Key words: hematology

ing is carried out by hematological experts using microscopy and using the XE-5000 hematological analyzer. **Results:** The hematological analyzer delivers more-accurate results with respect to normal cells but microscopy is suited better to recognize and count pathological cells. **Conclusion:** With the new hematological analyzers, several new hematological tests are made available as screening tools that were not possible previously using standard cell-counting procedures. J. Clin. Lab. Anal. 29:175–183, 2015. © 2014 Wiley Periodicals, Inc.

INTRODUCTION

Modern hematological analyzers combine the efficiency of the Coulter principle with the precision of flow cytometry, in contrast to the first hematological analyzers, which operated exclusively under the Coulter principle. This allows not only a quantitative determination of the three major blood cell types (erythrocytes, leukocytes, and platelets (PLT)) but also a quantification of subpopulations of the leukocyte lineage leading to an extended complete blood cell count (CBC). Furthermore, the combination of fluorescence flow cytometry with the fluorescent staining of nucleic acids within the cells allows a very good separation of the cell subpopulations that comprise the whole blood and permits the use of additional tests to further specify cellular blood components. Additionally, hematological analyzers that use this technology are able to detect immature cell populations in the blood. This technology is currently integrated into hematological analyzers by several manufacturers including Sysmex Corporation (Kobe, Japan) and is currently made available for use (1–5).

The ability to detect immature cells allows the detection of regeneration defects in individual cell populations earlier and faster than it is possible by exclusively analyzing

mature cells. Immature cell populations as well as several other cell population based parameters derive from calculations among the cells that are apparent in the fluorescence scattergrams. Here, the usage of fluorescence flow cytometry for CBC determination is discussed, and the novel cell population discriminatory parameters derived from the fluorescence scattergrams are presented (5). Furthermore, CBC determination by fluorescence flow cytometry is compared to manual blood cell count.

One hundred cells are typically characterized by a manual CBC, whereas modern hematological analyzers are capable of analyzing 7,000 to 10,000 cells in a brief period of time to yield both absolute and relative values of leukocyte subpopulations. Both methods, manual method versus fluorescence flow cytometric method, for CBC determination are compared in more detail. The sheer

*Correspondence to: Borros M. Arneth, Institute of Clinical Chemistry and Laboratory Medicine, Carl Gustav Carus University Hospital, University of Technology Dresden, Fetscherstr. 74, 01307 Dresden, Germany. E-mail: borros.arneth@uniklinikum-dresden.de

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processivity of modern hematological analyzers underscores the advantage of this technology over older, manual methods.

METHODS

First Method: Method of Fluorescence Flow Cytometry for Hematological Analysis

Overview of fluorescence flow cytometry in detail and method description

When blood cells are analyzed by fluorescence flow cytometry in an XE-5000 analyzer first, fluorescently labeled conjugates (polymethine dyes) capable of marking a specific blood cell epitope are incubated with the samples (5). After this labeling reaction, the blood cells are analyzed by flow cytometry. When passing through the flow cell, the cells are excited with monochromatic light from a semiconductor laser, which produces a characteristic wavelength of light that is detected. Depending on the scatter angle of the excitatory light detected, the cell counts of different cell populations can be determined from the resulting scattergrams. Different cell properties are capable of being exploited for the detection of specific cell populations.

The forward-scattered light is directly proportional to the cell size and is used as an indicator of the cell size. The side-scattered light indicates the internal cell structure and complexity and is proportional to the granularity of the cells. After nucleic acid staining with fluorescent dyes, the detected fluorescent light corresponds to the concentration of nucleic acids (sum of DNA (deoxyribonucleic acid) and RNA (ribonucleic acid)) within the cell.

The technology of the different channels

Fluorescence-based flow cytometry methodology

The WBC (white blood cell)/basophil granulocytes, basophil granulocyte channel (BASO), differentiation, differentiation channel (DIFF), and NRBC (nucleated red blood cell) channels are used for cell differentiation by flow cytometry. Before analyzing blood samples by flow cytometry, defining epitopes (DNA and RNA) of the blood cell subpopulations of interest are labeled with a fluorescently conjugated molecule. After the labeling reaction, the blood cells are analyzed by flow cytometry. When passing through the flow cell, the cells are excited with monochromatic light from a semiconductor laser, which produces a characteristic wavelength of light that is detected. Depending on the scatter angle of the excitatory light detected, the cell counts of different cell populations can be determined from the resulting scattergrams.

Upon excitation of the target fluorophore, the laser beam becomes diffracted to different extents and in dif-

ferent angles. The detected signals provide various pieces of information about the particular cell being analyzed, such as the following:

- the forward scattered light, which corresponds to the cell size;
- the side scattered light, which corresponds to the inner cell structure, complexity, and granularity;
- the lateral fluorescent light, which corresponds to the concentration of RNA and DNA in the cell when nucleic acid specific dyes are used to label the cells.

Therefore, by fluorescence flow cytometry, the following cellular characteristics are determined:

- cell size;
- internal structure of the cells, such as granulation;
- content of nucleic acids (RNA + DNA).

The red blood cells/PLT (RBCs/PLT) channel: Impedance measurement by means of hydrodynamic focusing (HDF)

RBCs and PLT are analyzed simultaneously in an impedance-measuring chamber because they can be distinguished based on their differences in size. A 4 μ l blood sample is diluted in a mixing chamber at a ratio of 1:500. This dilution is injected into the measuring chamber and is drawn through a capillary opening. When the cells pass through this aperture, they generate an electrical resistance change that registers as an electrical pulse according to the Coulter principle. The size of each electrical pulse is directly proportional to the size of the cell passing through the orifice. This device also measures the number of pulse changes for a predefined sample volume in a given amount of time.

With the help of HDF, the cells are surrounded by a cylindrical mantle flow that produces the cell sheath. The cell sheath ensures that all cells are centered in the aperture and pass single file, and it prevents signal interference that may arise from double passages (coincidences) or recirculating cells, thus ensuring an accurate cell count. Furthermore, the cell sheath is flushed continuously, effectively minimizing clogging that may occur.

The proportions of RBCs and PLT are then assessed in separate distribution curves based on their impedance values. The RBC/PLT channel is used to determine the hematocrit level, which is determined by the sum of all individual pulses. The RBC indices mean corpuscular volume, MCH (mean corpuscular hemoglobin), and mean corpuscular hemoglobin concentration are averaged over several measurements from individual cells.

The cellular distributions of RBCs and PLT are shown in separate histograms. The IRF and immature platelet fractions (IPFs) are determined using this channel. The

IRF is further subdivided into the low- (LFR), medium- (MFR), and high-fluorescing reticulocyte (HFR) fractions, which correlate with increasing fluorescence and immaturity.

The DIFF channel in fluorescence flow cytometry

The DIFF channel is used to quantify the remaining four cell populations of leukocytes: neutrophils, eosinophils, lymphocytes, and monocytes. Blood samples are diluted with the reagent Stromatolyser-4DL (Sysmex), which consists of a lysing reagent and a fluorescent dye in a ratio of 1:51. Stromatolyser-4DL lyses all RBCs during this process and perforates the cell membranes of the leukocytes, but it largely maintains the integrity of the leukocytes during the subsequent steps of the staining process. The cells are stained with the fluorescent dye, Stromatolyser-4DS, which is a polymethine fluorescent dye that penetrates the cell membrane and stains the nucleic acids in the nucleus and cytoplasm. Nucleic acid staining provides evidence regarding the activity level and maturity level of the cells based on the fact that activated and immature cells contain a higher level of nucleic acids.

The WBC/BASO flow cytometry channel

The total WBC and basophil counts are acquired via the WBC/BASO channel. The WBC and basophil counts require the dilution of a blood sample with the reagent Stromatolyser-FB in a 1:51 ratio. Stromatolyser-FB lyses all RBCs and causes acid-mediated shrinkage of the leukocytes. However, basophils remain unaffected by this treatment and can thus be differentiated. During flow cytometric analysis, the forward and side scatter are measured. Treatment with Stromatolyser-FB causes significant differences in the size and internal structure of leukocytes and basophils, which allows reliable separation of the WBC and basophil populations.

Results from the immature myeloid information (IMI) channel, which detects immature granulocytes (IGs), are compared with the results from the IG count obtained from the WBC DIFF channel.

The IMI channel: Using the radio frequency/direct current (RF/DC) method

The XE-5000 hematological analyzer includes an IMI channel, which is capable of identifying immature precursors of granulocytes and thus offers a second, independent measurement to verify the IG count obtained from the DIFF channel. The IMI channel is automatically included in the measurement of a CBC. Before the cells are analyzed, they are incubated with Stromatolyser-IM, which selectively lyses the mature leukocytes based on higher levels of cytoplasmic lipid content (the lipid content of cells increases with their maturity). In contrast, immature leukocytes contain proportionately more amino acids

than lipids in their cytoplasm; therefore, they are much less affected by the reagent. Following preparation, the sample is aspirated through a high-frequency radio transducer and is counted completely. Upon counting, the DC and RF signals are recorded, which reflect the volume (DC) and the ratio of nucleus to plasma (RF) of the cells. These results permit the discrimination of the cells into multinucleated granulocytes, IGs (promyelocytes, myelocytes, and metamyelocytes), and blasts. The IMI channel of the XE-5000 offers the unique ability to detect hematopoietic stem cells directly from the EDTA (ethylenediaminetetraacetic acid) sample tube without any additional sample preparation. The ability to detect an increase in hematopoietic progenitor cells (HPCs) can be useful to determine the optimum sampling conditions for stem cell preparations.

The RET channel: fluorescence flow cytometry

The reticulocytes (RET) channel permits the analysis of reticulocytes (Retis) and Reti precursors. To use the RET channel, blood samples are incubated with the reagent Ret-Search, which consists of a lysing reagent that perforates the membranes of all WBCs and a fluorescent dye that penetrates the cells and binds to the nucleic acids in the nucleus and cytoplasm. In the flow cell, the forward light scatter is plotted against the fluorescence intensity.

Leukocytes and erythroblasts have significantly higher nucleic acid content than other blood cells, which results in more-intense staining of these cells and a clearer separation from the Retis. In contrast, mature erythrocytes contain nearly no nucleic acids and can, therefore, be clearly distinguished from the Retis, which contain RNA. Furthermore, as Retis mature, their RNA content continuously increases, thereby facilitating the division of the Retis into three "age" groups:

- "mature" Retis (LFR);
- "semi-mature" Retis (MFR);
- "immature" Retis (HFR).

As the XE-5000 identifies the RBCs in this measurement channel via the forward light scatter, the hemoglobin (He) content of these cells is measured simultaneously as the new parameter reticulocyte hemoglobin (RET-He). The XE-RET master software is available for the XE-5000 to determine the He content of the Retis. Based on the detected He levels, the reticulocyte hemoglobin (Reti-He) content is determined as the RET-He value.

Procedure of testing the first method

Multiple day-to-day measurement repetitions of control material (hematological controls, Streck, Omaha, NE) have been carried out with the XE-5000 hematological analyzer.

Second Method: Method of Manual Microscopy

For the manual microscopy, slides were stained with the SP-1000 automated slide maker and dyer (Sysmex) using a Pappenheim cell staining.

CBCs have been carried out by microscopy (hand method) using an IFNAMED hematology Carl Zeiss microscope (50 $\times$ and 100 $\times$ objective; Carl Zeiss, Jena, Germany; 6,3 $\times$ ocular and 1,25 $\times$ blend) with oil immersion. Twelve independent hematology experts performed microscopy of blood smear independently and blinded.

RESULTS

At our institute in Dresden, Germany, we perform approximately 830 blood cell counts per day, which include $\sim$ 158 CBCs. From the CBCs performed in a given day, $\sim$ 39 are manually reviewed. This corresponds to a review rate of approximately 25% with the combined Sysmex XE-5000/Diffmaster DM 1200 CellaVision (Uppsala, Sweden) System. From Monday through Friday, $\sim$ 1,001 blood cell counts are performed each day, including $\sim$ 204 differential CBCs, of which $\sim$ 51 are manually reviewed. On weekends, our institution performs $\sim$ 515 blood cell counts per day, including $\sim$ 103 differential blood cell counts, $\sim$ 23 of which are manually reviewed (all average values).

The results of the comparison of the hand method (microscopy) and the Sysmex XE-5000 hematological analyzer are shown in the tables. Table 1 gives the results of the manual differentiation done by 12 hematology experts in more detail. A significant difference between different observers for the low-count cell populations has been found. Table 2 gives the results of 1-month daily measurements using the XE-5000 hematological analyzer. Table 3 gives an comparing overview over the different methods for CBC determination compared here.

It can be seen that the hematological analyzer has a significant higher accuracy in counting the known cell populations (normal cells). Furthermore the hands-on time is significantly lower, if the hematological analyzer is used. On the other hand, the hematology machine cannot categorize pathological cells at the moment. So, for pathological cells manual microscopy still is absolutely necessary. However, manual microscopy can be assisted by a camera and computer-supported method.

The main difference between the XE-5000 analyzer and the other hematological analyzers used before is a significant lower review rate. Using the older analyzer reviews had to be done according to several different reasons including Retis, errors concerning the PLT, left shift, and the occurring of pathological cells, while using the XE-5000 the review rate could be significantly reduced. Reviews are

necessary nearly solely according to pathological cells or left shift, besides some very infrequent other reasons.

The accuracy of automated Reti counting depends on the proportion of Retis in the sample. Therefore, smears with less than 2% Reti content are reviewed manually. Samples that contain a higher proportion of Retis ($>2\%$) are substantially more accurate when counted by a hematological analyzer due to the higher overall number of cells counted. Hematological analyzers can count up to 10,000 cells, whereas only approximately 1,000 cells can be processed through manual Reti counting. Using automated hematological analyzers, we are able to analyze over 99% of all Reti in a given sample, resulting in less than one manual Reti count per month.

DISCUSSION

Comparing Manual Microscopy and Modern Hematological Analyzers

According to the results of the method comparison here, clear advantages of the flow cytometrical hematological analyzer are a higher accuracy and a faster result. Disadvantages of the hematological analyzer are that not all cells, especially pathological cells can be determined correctly. Advantages of the manual method are that pathological cells as blasts and immature cells can be determined correctly and thereby leukemia and other tumor diseases can be detected. Therefore, at the moment only a combination of both methods can provide useful, fast, and correct results.

Taken together, at the moment, only a combination of both methods flow cytometrical automated CBC determination and manual CBC determination can provide fast and reliable results. Therefore, a positive effect is given by the relatively low review rate of the flow cytometric analyzer used here.

The manual slide evaluation cannot be replaced in total by flow cytometric automated methods at the moment because of the great variability of pathological cell types. Also, methods using pattern recognition systems combined with artificial intelligence, for example, the Diffmaster cannot—at the moment—correctly differentiate pathological cells and cannot therefore replace human hematology specialists.

The extended blood cell count can rapidly supply a large number of answers to important clinical hematological questions. The extended blood cell count represents an important diagnostic tool for the assessment of thrombocytopathies (TTP (thrombotic thrombocytopenic purpura), ITP (immune thrombocytopenia), hemolysis elevated liver enzymes low platelet syndrome (HELLP), HUS (hemolytic uremic syndrome), and DIC (disseminated intravascular coagulation)), erythropathies, and

TABLE 1. CVs of the CBC Counted by Manual Counting

	1	2	3	4	5	6	7	8	9	10	11	12	Mean	SD	CV%
Blasts	4	0	0	0	2	1	2	0	2	2	0	1	1.2	1.3	a
Promyelocytes	3	1	0	2	4	2	5	3	2	2	0	1	2.1	1.5	a
Myelocytes	7	6	7	5	8	4	7	3	8	7	15	7	7.0	3.0	42.2
Metamyelocytes	5	3	2	7	4	2	6	3	7	7	9	8	5.3	2.4	46.0
Bands	12	16	13	10	15	23	9	16	11	17	17	8	13.9	4.3	30.6
Neutrophils	44	46	49	50	41	41	37	44	42	36	43	41	42.8	4.2	9.8
Eosinophils	0	0	0	0	0	0	0	0	0	0	0	0	0.0	0.0	0.0
Basophils	0	0	0	1	0	0	0	0	0	0	1	0	0.2	0.4	a
Monocytes	16	13	13	18	19	15	22	22	17	23	11	21	17.5	4.0	22.9
Lymphocytes	1	9	8	7	7	12	7	9	11	4	4	0	6.6	3.7	56.3
Plasma cells	8	0	0	0	0	0	0	0	0	0	0	0	0.7	2.3	a
NRBCs	1	0	0	0	0	1	0	0	0	0	0	0	0.2	0.4	a
Diverse cells	0	6	8	0	0	0	5	0	0	0	0	0	1.6	2.9	a
SEM														2.3	29.7
Total count	102	102	103	104	105	107	107	108	109	108	111	99	99	33	

^aToo small cell counts for a statistical evaluation.

CVs of manual CBC done by 12 different experts. It can be seen that especially for cell populations with low cell counts (e.g., basophils, erythroblasts) the CV is high. On the other hand, determination of pathological cell types (diverse cells, tumor cells, plasma cells) is possible. Statistics are calculated with SPSS.

CV, coefficient of variation; SPSS, Statistical Package for Social Science.

leukopathies. Because a larger number of blood cells is counted, extended automated blood cell counts are substantially more precise than manual microscopic blood cell counts, which are still standard practice in most medical laboratories for the assessment of left shifts in the granulocyte population. Currently, the extended automated cell count cannot substitute for the microscopic cell count, especially for answering hematological questions regarding the presence of pathological cells, but it can be used as an auxiliary tool. It remains to be seen whether this new approach can improve hematological diagnostics.

The results of an automated blood cell count are based on the evaluation of 5,000 to 10,000 events. The exact number of events recorded can differ if the sample is not specified based on the leukocyte concentration. When manual counting is used, only 100 cells are differentiated and counted. Because automated cell counting takes into account many more events, this technique is typically more precise than manual counting. Manual blood cell counts are associated with several sources of error including distribution error due to disproportionate cell populations in the sample, subjective interpretation, and

TABLE 2. CVs of the CBC Counted With the Automatical Hematological Analyzer (Sysmex XE-5000)

	1	2	3	4	5	6	7	8	9	10	11	12	Mean	SD	CV%
Blasts (%)	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
Promyelocytes (%)	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
Myelocytes (%)	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
Metamyelocytes %	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
Bands %	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
Neutrophils %	44	45	45.5	45.3	45.2	46	46.2	45	44.5	43	43.5	46	46.2	1.0	2.2
Eosinophils %	9.3	8.9	9.2	9.1	9	8.9	10	10.7	11.1	8.7	9.9	9.6	8.7	0.8	8.7
Basophils %	4.9	4.8	4.5	4.4	5.2	4.6	4.3	5.6	5.6	5.6	4.3	4.6	5.7	0.5	9.0
Monocytes %	9.6	9.6	9.1	10.8	8.9	11.1	10.3	9.9	9.9	9.4	9.9	9.6	10.8	0.6	5.9
Lymphocytes %	36.4	36	36.9	36.5	34	38.4	35.6	35.4	36.4	32.8	37.1	36.4	37.9	1.5	3.8
Plasma cells %	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
NRBCs %	5.5	6.6	4.5	5.9	5.1	5	5.4	5.8	5.3	4.7	5.1	5.8	4.9	0.6	11.8
Diverse cells %	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
SEM														0.8	6.9
Total count	7,548	8,457	5,468	6,884	7,561	5,846	7,568	9,482	8,684	9,574	7,539	8,425	9,183		

^aThese values cannot be determined separately by using this hematological analyzer. However the promyelocytes, myelocytes, and metamyelocytes are summarized as IGs.

CV, coefficient of variation.

TABLE 3. Comparison of Old and New Methods for Counting Blood Cells

	Blood cell count by hand method	Old hematological analyzer	XE-5000 hematological analyzer	Diffmaster 1200
Counted cells	100	Up to 1,000	Up to 10,000	120
Time for one analysis	3–5 min	50 sec	40 sec	3 min
Hands-on time (per slide)	5–10 min	20 sec	10 sec	2–3 min
Documentation	No documentation	No documentation	Scatter plots	Photos of each cell
Evaluation	Microscope, “by hand”	Automatic evaluation	Automatic evaluation	Evaluation on the computer
Analytics	Human	Machine	Machine	Computer-assisted human
Manual microscopic review rate “by hand”	0	30–40%	10–30%	0–10%
Manual review necessary	0*	Pathological WBC, PLTs, RETI	Pathological slides only, left shift	Very few pathological slides
Suitable best for CV variation	Pathological samples 10–350%	Normal samples 0–20%	Normal samples 0–10%	Pathological samples 10–100%

Manual review rate: this terminus describes re-analysis necessary using the hand method/microscopy as done by hematology professionals. Re-analysis might be necessary due to system errors and/or due to abnormal cells (e.g., blasts, promyelocytes). System errors and abnormal cells normally lead to a flag indicating that a manual re-analysis is necessary.

*, manual review analysis is not necessary here.

small sample size. Small sample size associated with manual cell counting is one of the major obstacles overcome by modern hematological analyzers because automated analyzers are capable of analyzing and classifying a much larger number of cells. However, if pathological cells are differentiated, manual microscopy is still superior to a hematological analyzer, because the analyzer cannot recognize and rate pathological cells.

Automatic Blood Cell Counts in Absolute Values and/or Relative Values

Clinicians typically want to know blood cell counts as a percentage of the total population of blood cells. Although leukocytes arise from a common progenitor stem cell, they can be regarded as independent in terms of maturation, function, and regulation. Therefore, it is necessary to include the absolute values of cell populations in the automated differential blood cell counts. Only with the microscopic blood cell counts are the relative cell population numbers meaningful because only 100 cells are counted and categorized (6). A microscopic blood cell count should be requested only for a specific hematological question when the clinical picture is relatively clear (e.g., for the differentiation of leukemia or lymphomas).

Novel Parameters: The Extended Blood Cell Count

In recent years, fluorescence flow cytometry has enabled the identification of subpopulations of immature cells as well as the status of leukocyte activation. Knowledge of

the individual cell populations could provide important information with respect to clinical diagnosis and treatment. Therefore, these new parameters given by hematological analyzers have the potential to improve the clinical diagnostics. The most important new parameters can be divided into the extended RBC count and into the extended differential cell count.

Extended RBC Count

The extended small RBC count includes the IPF, HFRs, IMFRs, Reti-He, delta-hemoglobin (ΔHe), fragmentocytes (also called schistocytes, FRC), erythroblasts (NRBC), highly fluorescent lymphocytes (HFLCs), IGs, and activated granulocytes neutrophil scattering in y-axis (NEUT-Y). These new measurements may represent tools with which to diagnose SIRS (systemic inflammatory response) and sepsis.

The extended blood cell count already includes the IPF, which allows the reconstitution process of the bone marrow following chemotherapy or stem-cell transplantation to be followed (7). The Reti-He is a direct measure of the current iron availability. This parameter is lowered during acute or chronic inflammation. Likewise, Reti-He levels rise very rapidly after iron supplementation (after approximately 48 h) (8,9). This marker is important for monitoring dialysis patients undergoing erythropoietin therapy. Reti-He is also valuable for the distinction of a lack of iron (normal Reti-He levels) from iron deficiency as a result of anemia (decreased Reti-He levels) (10). This parameter could be useful for monitoring an iron substitution therapy.

The difference in He (ΔHe) is calculated as the difference between Reti-He and the average amount of He per adult erythrocyte (MCH) [$\Delta\text{He} = \text{Reti-He} - \text{MCH}$]. ΔHe is a measure of the difference in He concentrations between immature and mature erythrocytes. ΔHe can now be measured with the newest generation of fully automated hematological analyzers (11). The ΔHe value should always be positive, as Retis should contain more He than mature erythrocytes. Negative values indicate that the Retis no longer contain more He than the mature erythrocytes and suggest chronic anemia. During inflammation, interleukin-6 causes the release of hepcidin from the liver. Hepcidin binds to ferroportin and inhibits iron transportation; consequently, less iron and less He become available for biosynthesis in the Retis (12). Therefore, ΔHe values could be useful to differentiate between anemia due to chronic inflammation and anemia due to iron deficiency. Reti-He and ΔHe levels provide information regarding erythropoiesis during the previous 7 days. In contrast, the MCH and the percentage of hypochromic erythrocytes reflect erythropoiesis that occurred during the previous 8 to 12 weeks. This parameter could be useful for diagnosing an iron deficiency anemia and also for monitoring an iron substitution therapy.

FRCs result from mechanical damage to erythrocytes. FRCs can arise from mechanical stress, endocarditis, and microangiopathies. Microangiopathies can result from TTP, HUS, DIC, and stem-cell transplantation. The presence of FRCs requires immediate medical intervention (13). An automatic FRC measurement can also be used to effectively monitor the progression of microangiopathies or stem-cell transplantations. Normal values range from less than 0.1 to 0.6 FRCs per 1,000 erythrocytes (14). However, caution must be used in the interpretation of reported FRC levels as automatic counts may mistakenly label microcytes as FRCs.

Erythroblasts (NRBC) are typically only found in the peripheral blood of newborn children within the first week of life. Erythroblasts also represent potential prognostic markers for certain disease processes. In 2001, Stachon et al. described the prognostic significance of erythroblasts in the peripheral whole blood of patients who underwent cardiac surgery (15, 16). The presence or absence of erythroblasts as well as the absolute number of erythroblasts appears to correlate with increased mortality risk (17). One study showed that a concentration of more than 10,000 erythroblasts per liter in the circulating blood correlated with patient deaths in 100% of the cases analyzed (17). Erythroblasts in the whole blood of critically ill patients represent a transient, relatively early indication of a critical condition and help to rapidly estimate the mortality risk in certain patients. The presence of erythroblasts in the peripheral whole blood can also aid in the differentiation of sepsis from hemolytic

anemia. Therefore, knowledge of patient history is critical and, in the case of hemolytic anemia, knowledge of the haptoglobin, LDH, and potassium levels is critical in determining the extent of hemolysis. Erythroblasts are not typically present in healthy subjects; therefore, the NRBC levels of healthy subjects should be zero or close to zero. This parameter could be useful for monitor patients with life-threatening diseases.

Extended Differential Blood Cell Count

IGs consist of myelocytes, metamyelocytes, and promyelocytes, and they are counted within the IG gate. Blasts and bands are gated as separate populations and are counted separately. The IG gate includes all cells that are similar in size to neutrophils but contain higher nucleic acid content and thus fluoresce more intensely. IGs include activated reactive cell populations and immature cell populations. The IG level provides information about the activation status of the neutrophils, which is useful for the differential diagnosis of bacterial sepsis. Whereas microscopic differentiation can differentiate subpopulations within the IGs, hematological analyzers produce a single IG value that summarizes all immature cells (myelocytes, metamyelocytes, and promyelocytes). Therefore, it remains unclear whether this single value can replace microscopic cell differentiation. Patients with sepsis consistently present with increased IG levels. This parameter could be useful for monitoring patients with infections and/or sepsis.

Highly fluorescent lymphocytes (HFLCs) reflect the activated B-lymphocytes and the plasma cells in the peripheral whole blood. The HFLC level represents all cells that are close in size to neutrophils but have a higher nucleic acid content that results in increased fluorescence after staining. Therefore, the HFLC level is typically absolutely (HFLC#) and relatively (HFLC%) comparable to the IG level. HFLCs appear to correlate with high-titer viral infections and are, therefore, ideal markers with which to differentiate between infectious and noninfectious inflammatory conditions. Highly fluorescent lymphocytes are typically not present in healthy subjects; therefore, the HFLC values of healthy subjects should be zero or close to zero. Patients with sepsis have been shown to display dramatically elevated levels of HFLCs. This parameter could also be useful for monitoring patients with infections and/or sepsis.

The neutrophil activity marker "NEUT-Y" corresponds to the y -coordinate of the neutrophil population in the DIFF scattergram and quantifies the RNA content and phagocytic behavior of the neutrophils. NEUT-Y describes the y -values of the neutrophil population on the scattergram, which corresponds to the average fluorescence. Together with the HFLC level, the NEUT-Y

level can be used to differentiate between viral and bacterial infections because neutrophils are not involved in the viral immune response (10). The NEUT-Y level might possess substantially more value than the commonly used C-reactive protein (CRP) level, which reaches a maximum after 48 h. In addition, CRP possesses an extended half-life, which requires a long waiting period to determine the success of certain therapies. Thus, the Sysmex XE-5000 hematological analyzer offers new tools that can be used in diagnosing SIRS and septic infections. Analogously, the "NEUT-X" value corresponds to the *x*-coordinate of the neutrophil population in the DIFF scattergram and therefore to the granularity of the neutrophils. This parameter could be useful for diagnosing patients with myelodysplastic syndrome myelodysplastic syndrome (MDS).

The following flags are the most common and important warnings provided by the Sysmex XE-5000. Events are flagged when they do not fit predefined gates, as well as when they meet other criteria. Complex mathematical algorithms are used to determine whether events should be flagged. Cell populations that do not fall within any of the expected gates are predominantly judged based on the pattern of the signal that they emit. Furthermore, in these populations, the results of the individual channels are compared among each other. By using an advanced flagging methodology, only relatively few manual differentiations are necessary.

The IPF has a number of potential applications, such as, in the determination of whether thrombopenia results from consumption or is a defect in thrombopoiesis (18–20). This parameter could be useful for identifying patients with ITP. Furthermore, because the IPF level increases earlier than the thrombocyte count, the IPF level can be used to monitor the hematopoietic activity in bone marrow transplant patients and newborns.

CONCLUSION

Currently, fully automated, hematologic analysis is the fastest type of analysis readily available in the medical laboratory. However—at the moment—only a combination of different methods including manual microscopy of pathological slides can ensure a high quality of the results. This is given by the fact that so far machines cannot reliably detect and differentiate pathological cells. Therefore, up until now, the human specialist is not replaceable. However, approaches based on pattern recognition and artificial intelligence make great progresses here and can at least support manual microscopy.

As discussed, the extended parameter spectrum of hematological analyzers provides several interesting new parameters to medical laboratories and clinicians. Therefore, at the moment, the usefulness of these extended parameters is not clear. Additional clinical studies are

needed to investigate the usefulness of these new parameters in more detail.

ABBREVIATIONS

BASO	= basophil granulocytes, basophil granulocyte channel
CBC	= complete blood cell count
CRP	= C-reactive protein
DC	= direct current
DIC	= disseminated intravascular coagulation
DIFF	= differentiation, differentiation channel
DNA	= deoxyribonucleic acid
FRC	= fragmentocyte
HDF	= hydrodynamic focusing
He	= hemoglobin
HELLP	= hemolysis elevated liver enzymes low platelet syndrome
HFRs	= high-fluorescing reticulocytes
HFLC	= high-fluorescing lymphocyte cells
HPC	= hematopoietic progenitor cells
HUS	= hemolytic uremic syndrome
IGs	= immature granulocytes
IMI	= immature myeloid information
IPF	= immature platelet fraction
ITP	= immune thrombocytopenia
LFR	= low-fluorescing reticulocytes
MCH	= mean corpuscular hemoglobin
MCHC	= mean corpuscular hemoglobin concentration
MCV	= mean corpuscular volume
MDS	= myelodysplastic syndrome
MFR	= medium-fluorescing reticulocytes
NRBC	= nucleated red blood cell
NEUT-Y	= neutrophil scattering in y-axis
PLT	= platelets
RBC	= red blood cells
RET	= reticulocytes
RET-He	= reticulocyte hemoglobin
Retis	= reticulocytes
Reti-He	= reticulocyte hemoglobin
RF	= radio frequency
RNA	= ribonucleic acid
SIRS	= systemic inflammatory response syndrome
TTP	= thrombotic thrombocytopenic purpura
WBC	= white blood cell
ΔHe	= delta-hemoglobin

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