Validation of Gating and Leukocyte Classification on Sysmex XE Series Automated Cell Counters

Mari KONO[*1], Tamiaki KONDO[*1], Yuri TAKAGI[*1], Atsushi WADA[*1], Ian GILES[*2] and Kunihiro FUNAKOSHI[*1]

[*1] *Cell Analysis Center, Scientific Affairs, Sysmex Corporation, 4-4-4 Takatsukadai, Nishi-ku, Kobe 651-2271, Japan.*

[*2] *Scientific Affairs, Sysmex America, Inc., 1 Nelson C. White Parkway, Mundelein, IL 60060, U.S.A.*

Corresponding Author: Mari Kono, 4-4-4 Takatsukadai, Nishi-ku, Kobe 651-2271, Japan E-mail: Kono.Mari@sysmex.co.jp

Leukocyte classification on Sysmex XE series hematology analyzers involves staining leukocytes with proprietory Polymethine Dye reagents that have affinity for nucleic acids. The outputs are two-dimensional scatter diagrams of side scatter and fluorescence intensity (DIFF scattergrams) subsequent to flow cytometric analysis of the stained cells. Leukocyte classification is based on the location of treated cells in the DIFF scattergram. Comparative methods for leukocyte typing can be done using monoclonal antibodies to CD antigens expressed on the cell surfaces.

The present study investigates the relationship between cell-classification by means of CD antibodies versus cell classification on the Sysmex hematology analyzer. This was achieved by double staining cells with both CD antibodies and Sysmex cell counting reagents.

Granulocytes, T and B lymphocytes, and monocytes were prepared using density gradient centrifugation and negative selection using magnetic cell sorting methods. Samples were peripheral blood samples from healthy human subjects. Isolated cells were stained with FITC-conjugated CD antibodies. Subsequently the same cells were incubated in Sysmex cell counting reagents in a way that mimics conditions used in the hematology cell counters. Post treatment, the cells were analyzed by flow cytometry (FCM), confocal laser scanning microscopy (CLSM), and electron microscopy (EM).

FCM analysis revealed that most CD3- and CD19-; CD14-; and CD16b-positive cells appeared in the lymphocyte; monocyte; and neutrophil gates on DIFF scattergrams. CLSM and EM observation shows characteristic morphological changes for each type of leukocyte, related to the degree of membrane disruption and nucleic acid distribution within the various cell types.

This experimental approach appears to validate the method for classification of leukocytes on DIFF scattergrams versus classification on the basis of surface antigen labeling.

Key Words Hematology Analyzer, Leukocyte Classification, CD Antibody, Flow Cytometry, Double Staining

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INTRODUCTION

In 1891, Ehrlich found that leukocytes in peripheral blood include various cell types which exhibit morphological differences[1)]. Presently, leukocytes in peripheral blood are classified into 5 types, including neutrophils, eosinophils and basophils as granulocytes, and lymphocytes and monocytes as mononuclear cells[2)], 100 years after Ehrlich's finding. The ratio of each type of leukocyte to all leukocytes is very important in clinical diagnosis of various types of inflammation, leukemias, and allergic reactions.

For example, neutrophils mobilize and increase in bacterial inflammation because their primary function is phagocytosis of bacteria, while they remain unchanged or decrease in viral inflammation because of aggregation due to complement activation or destruction by antibodies. The ratio of monocytes increases in various infections because monocytes play roles in inflammatory reactions, perform phagocytosis of bacteria, and present antigens to lymphocytes. The decline of lymphocytes causes immunodeficiency because lymphocytes play important roles in humoral immunity. In both leukemias and allergic reactions leukocyte numbers increase.[2-8)].

Thus, leukocytes have characteristic phenotypes because their individual roles *in vivo* depend on leukocyte type. Their morphological structures and the proteins they express depend on leukocyte type. Monoclonal antibodies (CD antibodies) to specific proteins (CD antigen) expressed on the surface of leukocytes are

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generally used to specifically classify leukocytes by flow cytometry[9,10].

Manual microscopic methods of leukocyte classification in clinical laboratories utilize morphological differences among leukocytes. For example, May-Giemsa-stained blood smear preparations obtained by the wedge method are observed under a microscope and classified by feature of morphological structure of the nucleus and cytoplasm, as well as affinity to neutral, basic, or acidic dye. Two skilled technicians count 200 leukocytes, and the mean values obtained are considered correct[11].

On the other hand, use of automated hematology analyzers, which can classify the 5 types of leukocytes, has became very widespread. Most of them adopt the principle of flow cytometry and classify leukocytes into 5 types using optical, electrical, and cytochemical properties[12]. The automated hematology analyzer, XE series (Sysmex, Kobe, Japan), exposes leukocytes to a specific reagent, STROMATOLYSER-4DL (Sysmex), containing surfactant, and STROMATOLYSER-4DS (Sysmex), containing fluorescent dye, displays two-dimensional scatter diagrams (DIFF scattergrams) based on fluorescence intensity and side scatter, and classifies leukocytes based on location in the DIFF scattergram[13, 14].

The principal method of examining the validity of results obtained with a hematology analyzer had been comparison with independent results obtained with a standard method using blood smear preparations or by typing using CD antibodies, on the same samples [15-21]. In this study, we directly compared classification of hematology analyzer with typing of CD antibodies at each cell level by double-staining the same leukocytes with CD antibody and Sysmex reagents, and analyzed the DIFF scattergram which was produced by an independent commercially available flow cytometer. Moreover, we examined the same leukocytes by confocal laser microscopy and Transmission electron microscopy, and speculated on the effects of the morphological differences of each leukocyte group represented in the DIFF scattergram.

MATERIALS AND METHODS

Blood

Peripheral blood from 5 healthy human subjects (3 female, 2 male; by venipuncture, with informed consent) was collected in tubes containing EDTA (Terumo, Tokyo, Japan). The mononuclear-cell-rich fraction and the granulocyte-rich-fraction were prepared with density gradient centrifugation according to the manufacturer's instructions using two types of Lymphocyte Separation Solution, with d = 1.077 and d = 1.119 (Nacalai Tesque, Kyoto, Japan) and washed with PBS.

Magnetic Cell Sorting (MACS)

T lymphocytes, B lymphocytes, and monocytes were isolated from the mononuclear-cell-rich fraction by negative selection with the MACS system (STEMCELL Technologies, British Columbia, Canada) according to the manufacturer's instructions.

Cell Staining

The granulocyte-rich fraction and isolated T lymphocytes, B lymphocytes, and monocytes were incubated with FITC-conjugated CD16b (neutrophil marker), CD3 (T lymphocyte marker), CD19 (B lymphocyte marker), and CD14 (monocyte marker) monoclonal antibody (DAKO, Glostrup, Denmark) solution at a concentration of 20 mg/L in PBS for 30 min at 4 °C. FITC-conjugated mouse IgG1 antibody (DAKO) was used as a negative control. After washing with PBS, cells were exposed to STROMATOLYSER®-4DL and STROMATOLYSER®-4DS in the same fashion as for the Sysmex XE-2100 as follows: 18 µL of sample, 882 µL of STROMATOLYSER®-4DL, and 18 µL of STROMATOLYSER®-4DS.

Flow cytometry

Stained cells were analyzed using a FACSCalibur(tm) (BD Biosciences, Franklin Lakes, NJ, USA).

Confocal laser microscopy

Stained cells were immediately attached to poly-L-lysine (SIGMA, Missouri, USA)-coated coverslips and observed with a confocal laser scanning microscope system (IX81, Olympus, Tokyo, Japan; CSU-X1, Yokogawa Electric, Tokyo, Japan; ImagEM, Hamamatsu Photonics, Hamamatsu, Japan).

Transmission electron microscopy

Stained cells were fixed in 1% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) /PBS for 16 h at 4 °C. Fixed cells were attached to silanized glass slides using Cytospin (Thermo Fisher Scientific, MA, USA) and post-fixed in 1% osmium tetroxide for 30 min at 4 °C. Following osmium fixation, the samples were dehydrated in a graded series of ethanols and inversion-embedded in Quetol 812 (Nisshin EM, Tokyo, Japan). The samples were cut into 80-100 nm sections with the Ultracut UCT ultramicrotome (Leica Microsystems, Wetzlar, Germany) and observed with the H-7500 transmission electron microscope (Hitachi High-Technologies, Tokyo, Japan).

RESULTS

First, the granulocyte-rich fraction, T lymphocytes, B lymphocytes and monocytes double-stained with FITC-conjugated CD antibodies and specific reagent were analyzed by flow cytometry to determine the relationship between typing by CD antibodies and classification by DIFF scattergram. The DIFF scattergram was reproduced with the STROMATOLYSER-4DS fluorescence intensity as the vertical axis and side scatter intensity as the horizontal axis using FACSCalibur (***Fig. 1A***), based on the DIFF scattergram obtained with Sysmex XE-2100 measurement of the same whole blood (***Fig. 1B***). The

granulocyte-rich fraction stained with CD16b-FITC (FITC-conjugated CD16b monoclonal antibody) was analyzed with the reproduced DIFF scattergram, with the vertical axis changed to FITC fluorescent intensity (***Fig. 1C***). Cluster of cells strongly stained with FITC was gated in comparison with those stained with negative control antibody (***Fig. 1C***) and analyzed with the reproduced DIFF scattergram in which the vertical axis was STROMATOLYSER-4DS fluorescence intensity (***Fig. 1D***). Cluster of cells strongly stained with CD16b-FITC, i. e. CD16b-positive cells, appeared at the site of neutrophils in the DIFF scattergram reproduced by FACSCalibur. T lymphocytes stained with CD3-FITC (***Fig. 1EF***), B lymphocytes stained with CD19-FITC (***Fig. 1GH***), and monocytes stained with CD14-FITC ***(Fig. 1IJ***) were analyzed in a fashion similar to the granulocyte-rich fraction. It is revealed that both of CD3 and CD19 positive cells were appeared at the site of lymphocytes, and CD14 positive cells were located at the site of monocytes in the reproducted DIFF scattergram.

Next, neutrophils stained with CD16b-FITC (***Fig. 2A***), T lymphocytes stained with CD3-FITC (***Fig. 2B***), B lymphocytes stained with CD19-FITC (***Fig. 2C***), and monocytes stained with CD14-FITC (***Fig. 2D***) were observed for patterns of staining with STROMATOLYSER-4DS using confocal laser microscopy (***Fig. 2A-D***). Neutrophils showed STROMATOLYSER-4DS stain to be distributed around the nucleus, cytoplasm and organelles (particularly in sites of nucleic acid rich organelles) (***Fig. 2A***). Staining patterns in T lymphocytes and B lymphocytes were very similar, with staining of the nucleolus and cytoplasm (***Fig. 2BC***). Monocytes also showed characteristic stain distributions (***Fig. 2D***). Cellular stain distribution patterns of STROMATOLYSER-4DS were characteristicly related to leukocyte sub-types (***Fig. 2E***).

Finally, leukocytes were observed for ultrastructural morphology using transmission electron microscopy. The granulocyte-rich fraction mainly contained granulocytes and erythrocytes (***Fig. 3A***), while isolated B lymphocytes (***Fig. 3B***), T lymphocytes (***Fig. 3C***), and monocytes (***Fig. 3D***) mainly contained each leukocytes. High-magnification images of ***Fig. 3A-D*** are shown in ***Fig. 3E-H***. Representative images of each type of leukocyte after treatment with specific reagent are shown in F***ig. 3I-L***. These images reveal that T lymphocytes (***Fig. 3J***), B lymphocytes (***Fig. 3K***), and monocytes (***Fig. 3L***) were, as mononuclear cells, disrupted more than granulocytes (***Fig. 3I***), and that morphological changes of the nucleus and the distribution pattern of organelles was dependent upon leukocyte sub-type.

DISCUSSION

Automated hematology analyzers have made possible rapid and easy leukocyte classification using low-cost surfactant and staining dyes without expensive antibodies. Many types of automated hematology analyzers have been developed by many companies. Result validation is typically done by comparison with other methods, including optical microscopy, or specific identification using CD antibodies and flow cytometry[15-21] . In the present study, the validity of the DIFF scattergram on the Sysmex XE-2100 was investigated by double-staining of the same cells with CD antibodies and Sysmex cell counter reagents, and conducting flow cytometric analysis. It was found that classification on

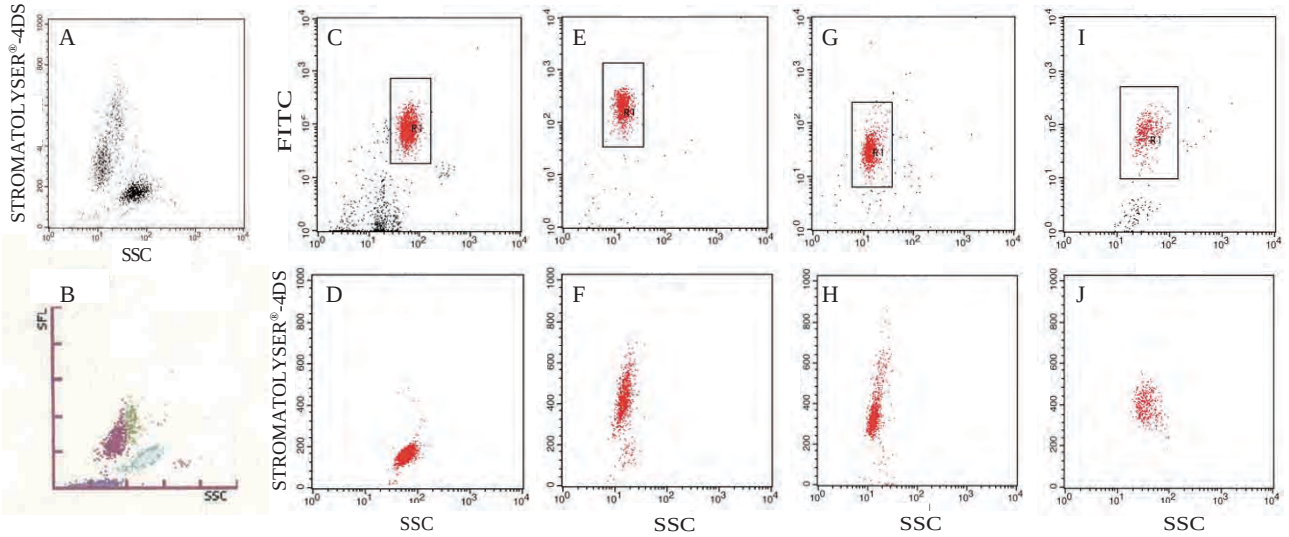

Fig. 1 *Flow cytometric analysis*

DIFF scattergram was reproducted by setting the STROMATOLYSER-4DS fluorescence intensity on the vertical axis and side scatter intensity as the horizontal axis on the FACSCalibur™ (A). The DIFF scattergram was obtained from Sysmex XE-2100 measurement of the same whole blood (B). Granulocyte-rich fraction stained with CD16b-FITC was analyzed by reproduced DIFF scattergram with the vertical axis changed to FITC fluorescence intensity (C). Cluster of cells strongly stained with FITC compared with negative control antibody (data not shown) were gated (C) and analyzed by reproduced DIFF scattergrams in which the vertical axis was STROMATOLYSER-4DS fluorescence intensity (D). T lymphocytes stained with CD3-FITC (EF), B lymphocytes stained with CD19-FITC (GH), and monocytes stained with CD14-FITC (IJ) were analyzed in a fashion similar to the granulocyte-rich fraction (CD).

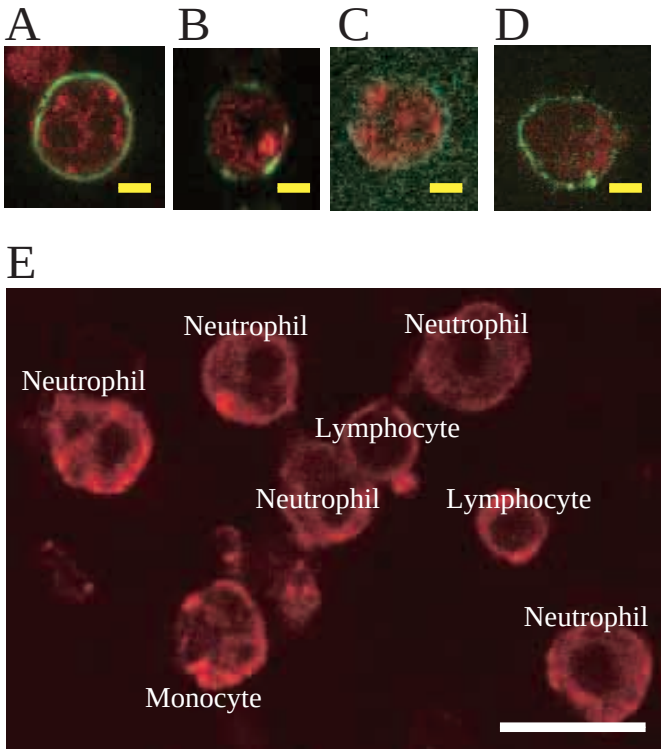

Fig. 2 *Confocal laser microscopic observation with double staining*

Neutrophil (A), T lymphocyte (B), B lymphocyte, (C) and monocyte (D) were stained green with CD16b-FITC, CD3-FITC, CD19-FITC, and CD14-FITC on their surfaces. They were stained red with STROMATOLYSER-4DS in characteristic fashion depending on leukocyte type. Granulocyte-rich-fraction (E) was stained with negative control antibody and specific reagent. White letters are expected leukocyte type according to staining pattern. Yellow Bar = 2 µm, White Bar = 10 µm

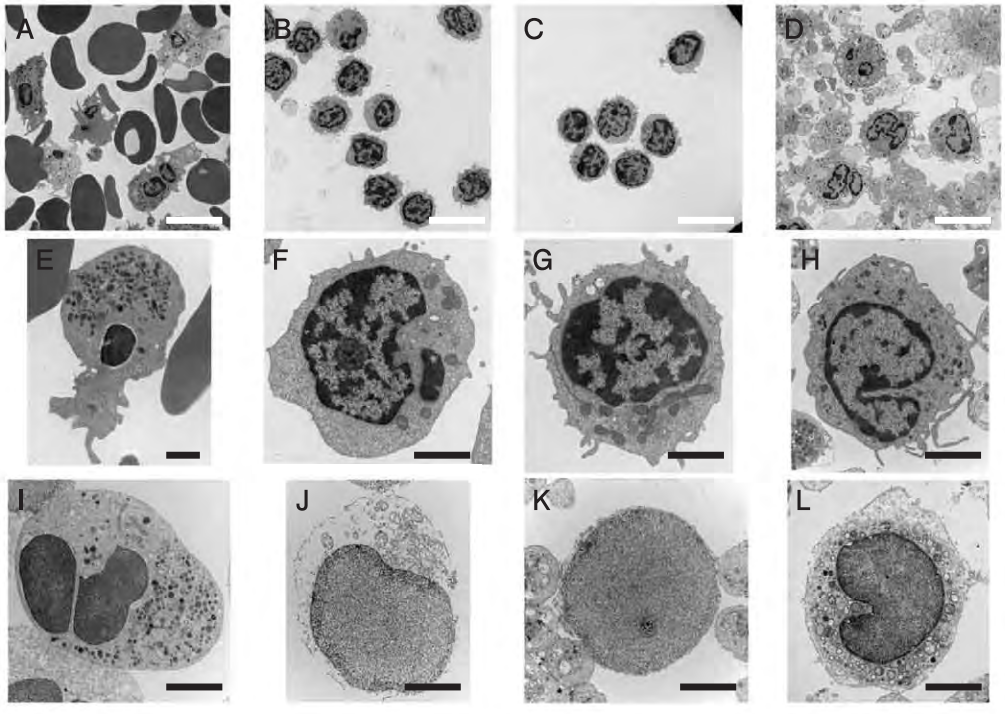

Fig. 3 *Electron microscopic observation*

Granulocyte-rich fraction (A), T lymphocytes (B), B lymphocytes (C), and monocytes (D). High-magnification images of (A)-(D) are shown in (E)-(H). Representative images of each type of leukocyte after treatment with specific reagent (I)-(L). White Bar = 10 µm, Black Bar = 2 µm

the DIFF scattergram agreed with typing using CD antibodies for each cell type. Moreover, the staining pattern of the specific reagent was strongly characteristic for each leukocyte sub-type on confocal laser microscopy. Differences were sufficient to predict leukocyte type from the staining pattern of cell counter reagents even in cases of leukocytes stained with negative control-FITC antibody (***Fig. 2E***). Differences in fluorescence patterns among types of leukocytes reflect the differences of fluorescence staining intensity and side scatter intensity, and produce cluster for each leukocyte type on the DIFF scattergrams.

Furthermore, after treatment with specific reagents, the leukocytes exhibited disruption of surface membranes for porosity enhancement. Internal organelles were largely preserved, depending on leukocyte type, as shown using transmission electron microscopy. Membrane disruption in mononuclear cells is more pronounced than for granulocytes. We reveal the effect of these differences in ultrastructural morphology by confocal laser microscope. It appears that the degree of the effect of surfactant on cell membrane relates to the degree of the introduction of staining dye, and thus it reflects the difference of fluorescence intensity among leukocytes in the DIFF scattergram.

Within leukocyte cell membranes, there are subdomains called lipid rafts which are rich in glycosphingolipids (GSLs) and cholesterol. Lipid rafts are believed to participate in signal transduction and are refered to as detergent-resistant membrane (DRM) due to the fact that they are insoluble in nonionic surfactants like TritonX-100[20, 24]. It has been determined that the proportion of lipid rafts varies depending on leukocyte sub-type, since leukocytes have specific features consistent with their roles *in vivo*. It is possible that the proportion of lipid rafts in each leukocyte type contributes to leukocyte classification on the DIFF scattergram.

Additionally, there are reports that changes in cell condition affect lipid rafts. Change in the cytoskeleton of neutrophils is influenced by lipid rafts[22]. Recruitment of lipid rafts occurs at sites of cell-cell contact between T cells and antigen-presenting cells following T receptor signaling[23, 24]. Monocytes stimulated by agonist have higher resistance to surfactant than unstimulated monocytes[25-27]. There are possibilities for monitoring drug sensitivity or immunological responses using the change of resistance to surfactant and morphological properties by setting the conditions of treatment by surfactant.

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