
Comparison of the Leukocyte differentiation Scattergrams Between the XN-Series and the XE-Series of Hematology Analyzers

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The newly launched XN-Series multiparameter, automated, hematology analyzer features a new channel named the WDF channel. Like the DIFF channel of the XE-Series, this channel can differentiate leukocytes from cells treated with specific reagents containing detergents and fluorescent stains, by using the 2-parameter flowcytometric method. The scattergrams of the 2 channels have different patterns due to the differences in the reagents used as well as differences in the hardware and software. In particular, the WDF channel differentiates between lymphocytes and monocytes and enhances the separation capacity, thereby distinguishing it from the DIFF channel. In this study, we morphologically examined the reasons for the positional appearance of each subtype of the leukocytes on the scattergram. Additionally, we also assessed the reason why lymphocytes and monocytes separated evidently on the WDF scattergram, in terms of the influence of the reagents.

First, using the XN- and XE-analyzers, we confirmed that the lymphocytes and monocytes separated better on the WDF scattergram than on the DIFF scattergram. Next, the separation of leukocytes was assessed following treatment with the WDF or DIFF reagents by the same method using the analyzers. Fluorescence staining was performed, and the intensity of the stained area in the leukocytes was observed under the Confocal Laser Scanning Microscope (CLSM) ; the intracellular structure of the leukocytes was observed under the Transmission Electron Microscope (TEM) ; and the size and surface structures of the leukocytes were observed under the Scanning Electron Microscope (SEM) . Each leukocyte appeared at the same position as they are measured in whole blood. Analysis under the CLSM showed that of all the leukocytes, the staining intensity after treatment with the reagents was highest in the monocytes, followed by that in the T lymphocytes, B lymphocytes, neutrophils, and eosinophils, which correlated with the side fluorescence intensity on the WDF scattergram. In addition, observation by TEM revealed that of all the subtypes of leukocytes, the intracellular complexity after treatment with reagents was simplest in the lymphocytes, followed by that in monocytes, neutrophils, and eosinophils, which correlated with the side-scattered intensity on both the scattergrams. Moreover, observation by SEM demonstrated that after treatment with reagents, the size of the lymphocytes was the smallest, followed by that of monocytes, neutrophils, and eosinophils, which correlated with the forward-scattered intensity on the WDF (FSC and SSC) scattergram. These observations indicated that each leukocyte cluster was different in terms of the amount of its organelles as well as the detergent tolerance of cell membranes.

From each electron microscope observation, it was clear that the intracellular structures of the leukocytes were retained after treatment with WDF reagents compared with that after treatment with DIFF reagents. In conclusion, the separation of lymphocytes and monocytes was better demonstrated by a WDF scattergram than by a DIFF scattergram.

Key Words XN-Series, WDF Channel, Separation Ability, Blood Cell Morphology

INTRODUCTION

Leukocytes play important roles in body defenses. Between 4,000 and 9,000 leukocytes are contained in 1 μ L of blood. In clinical laboratories, following the

recommended method from the Clinical and Laboratory Standards Institute (CLSI method H20-A2), a smear specimen stained with May-Giemsa dye is used to classify leukocytes into 5 groups: neutrophils, eosinophils, and basophils in the granulocytic fraction;

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and lymphocytes and monocytes in the mononuclear cell-fraction¹. Granular cells have a low nucleus to cytoplasm ratio and segmented nuclei. Neutrophils play a role in acute inflammation and make up 50% to 70% of the leukocytes found in the peripheral blood; they also have a variety of granules. Eosinophils increase with chronic allergies and parasite infections, and make up 2% to 5% of all leukocytes; they have nuclei shaped like kidneys and characteristic granules. Basophils are related to allergic reactions and make up less than 1% of all leukocytes; they are slightly smaller than neutrophils and have characteristic large granules. Monocytes make up 3% to 6% of all leukocytes, phagocytose foreign bodies, mainly become macrophages, and mostly have segmented nuclei. Lymphocytes play a central role in immunity, make up 40% of all leukocytes, have a large nucleus to cytoplasm ratio, and are mainly classified as T lymphocytes, B lymphocytes, and natural killer (NK)-lymphocytes. It is very difficult to classify these subtypes based on morphology; hence, it is necessary to check the classification by surface antigens. Notably, leukocytes can be classified by flow cytometry using antibodies that detect cell surface antigens (CD antibodies). The standard protocol for leukocyte differentiation, which was investigated by the CLSI, specifies that T lymphocytes, B lymphocytes, monocytes, and neutrophils express CD3, CD19, CD14, and CD16, respectively. Eosinophils are negative for the CD123 antigen and positive for the CD294 antigen³. Unfortunately, the method for classification using CD antigens is expensive and is a complicated procedure.

Therefore, in the clinical laboratory, automated hematology analyzers are used to classify leukocytes, which are quick and simple. Automated hematology analyzers use the principle of flow cytometry with specific reagents².

Sysmex Corporation launched the XE-Series automated hematology analyzer (XE-Series; Sysmex Corporation, Kobe, Japan) in 1999 and the XN-Series automated hematology analyzer (XN-Series; Sysmex Corporation) in 2011. These automated hematology analyzers have a leukocyte differentiation function that is called the DIFF channel (XE-Series) or the WDF channel (XN-Series). In these channels, a surfactant in specific reagents causes hemolysis and dissolution of red blood cells and platelets and differentially disrupts the cell membrane of white blood cells. Then, the fluorescent dye in the specific reagent enters the cells and stains the nucleic acid. The intensity of side fluorescent light (SFL) and side-scattered light (SSC) is measured using the principle of flow cytometry through excitation by a 633 nm laser beam. The results from this are described on a 2-dimensional scattergram³⁻⁵. Moreover, in the XN-Series, the forward-scattered light (FSC) is added, and is

described in a SSC-FSC scattergram.

The WDF channel can separate lymphocytes and monocytes better than the DIFF channel, but this has not been reported with morphological data. We used normal leukocytes to study the morphological examination results of the different leukocyte differentiation methods. We also assessed why lymphocytes and monocytes were more clearly separated on the WDF scattergram and relate that to the technological differences.

MATERIAL AND METHODS

1. Samples

Peripheral blood samples from 15 healthy human subjects were obtained by venipuncture and collected in tubes containing EDTA-2K (Terumo Corporation, Tokyo, Japan). Subjects gave their written informed consent before participation.

2. Cell separation

1) Density-gradient centrifugation

Peripheral blood samples from healthy volunteers were diluted in an equal volume of PBS. According to the manufacturer's instructions, blood was overlaid on 2 types of lymphocyte separation solution ($d = 1.077$ and $d = 1.119$) (NACALAI TESQUE, INC., Kyoto, Japan), and the mononuclear cell-rich and granulocyte-rich fractions were prepared by density-gradient centrifugation and washed with PBS.

2) Magnetic cell sorting

B lymphocytes, T lymphocytes, and monocytes were isolated from the mononuclear cell-rich fraction, and neutrophils and eosinophils were isolated from the granulocyte-rich fraction by negative selection with the RoboSep system (in the order, ST-19051, ST-19054, ST-19059, ST-19257, ST-19256; STEMCELL Technologies Inc., Vancouver, BC, Canada) according to the manufacturer's instructions⁶⁻⁷. Then, in order to recover the morphology observed in the peripheral blood, isolated cells were cultured in 1% albumin from bovine serum further purified Fraction V (A3294-100G; Sigma-Aldrich Corp., St. Louis, Missouri, USA) in PBS for 1 h at 37°C.

3. Confirmation of the purification of isolated cells

Isolated B lymphocytes, T lymphocytes, monocytes, neutrophils, and eosinophils were incubated with FITC-conjugated monoclonal antibodies to CD19 (B

lymphocyte marker, A07768; Beckman Coulter, Inc., Pasadena, CA, USA), CD3 (T lymphocyte marker, A07746; Beckman Coulter, Inc.), CD14 (monocyte marker, F0844; DAKO Denmark A/S, Glostrup, Denmark), CD16b (neutrophil marker, IM2353U; Beckman Coulter, Inc.), CD123 (first eosinophil marker, 130-090-897; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), and PE-conjugated monoclonal antibody to CD294 (second eosinophil marker, 120-001-698; Miltenyi Biotec GmbH). All antibodies were used at a concentration of 20 mg/L in PBS. The cells were incubated for 20 min at 4°C in the dark. FITC-conjugated mouse IgG₁ antibody (A07795; Beckman Coulter, Inc.) and PE-conjugated rat IgG₂ antibody (553930; BD Biosciences, Franklin Lakes, New Jersey, USA) were used as negative controls. Stained cells were washed with PBS and analyzed using a FACSCalibur™ (BD Biosciences), and the purity of isolated cells was then verified.

4. Measurement by XE-Series and XN-Series

Peripheral blood and isolated cells were measured using the XE-2100 multi-parameter automated hematology analyzer and the XN-2000 multi-parameter automated hematology analyzer, and cell positions on the scattergrams were then confirmed.

5. Treatment with specific reagents

Isolated cells were treated with XE-2100 specific reagents (Stomatolyser®-4DL, Stomatolyser®-4DS; Sysmex Corporation) and XN-2000 specific reagents (Lysercell™ WDF, Fluorocell™ WDF; Sysmex Corporation), with dilution rates and reaction times similar to those used with the XE-2100 and XN-2000 analyzers.

6. Confocal laser scanning microscopy (CLSM) analysis

Stained cells were immediately attached to poly-L-lysine (513-74891; Sigma-Aldrich Corp.)-coated coverslips, and the staining position of cells with the WDF-specific reagent was observed using a confocal laser scanning system (IX81; Olympus Corporation, Tokyo, Japan, CSU-X1; Yokogawa Electric Corporation, Tokyo, Japan, Imagem; Hamamatsu Photonics K.K, Hamamatsu, Japan). The fluorescence intensity of each leukocyte (N > 80) was measured from these images, and mean values

and error bars were calculated.

7. Electron microscopy analysis

1) Cell fixation

Each cell preparation was fixed in a 1.5% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) solution in PBS for at least 16 h at 4°C.

2) Examination by transmission electron microscopy (TEM)

Fixed cells were attached to MAS-coated glass slides using a Cytospin® (Thermo Fisher Scientific Inc., Waltham, MA, USA) and post-fixed in 1% osmium tetroxide for 45 min at room temperature (RT). Following osmium fixation, the samples were dehydrated in a graded series of ethanol solutions and inversion-embedded in Quetol 812 (Nisshin EM Corporation, Tokyo, Japan). The samples were cut into 60 to 80 nm sections with an Ultracut UCT ultramicrotome (Leica Microsystems GmbH, Wetzlar, Germany) and stained with uranyl acetate (94260; Wako Pure Chemical Industries, Ltd. Osaka, Japan) and lead water (99723-64; NACALAI TESQUE, INC.). Fixed cells were observed with the H-7500 TEM (Hitachi High-Technologies Corporation, Tokyo, Japan).

3) Examination by scanning electron microscopy (SEM)

Fixed cells were attached to poly-L-lysine-coated glass slides and post-fixed in 1% osmium tetroxide for 45 min at room temperature (RT). Following osmium fixation, samples were dehydrated using the same method as described above for TEM, but ethanol was replaced with t-butyl alcohol (028-03386; Wako Pure Chemical Industries, Ltd.). The samples were then dried using a freeze-dryer ES-2030 (Hitachi High-Technologies Corporation) and osmium-coated using Neoc (MEIWAFOFOSIS CO., LTD, Tokyo, Japan). The samples were then observed using a JSM-7500F system (JEOL Ltd., Tokyo, Japan).

8. Observation by 3D-laser microscope

Leukocytes used for SEM samples in 7-3) were observed by 3D-laser microscope (LEXT-OLS4000; Olympus Corporation). Cross sections were measured for at least 100 cells, and the area of maximum, center, and minimum data were calculated and indicated with graphs using an annexed application.

RESULTS

1. Position of the isolated leukocyte subtypes

T lymphocytes, B lymphocytes, monocytes, neutrophils, and eosinophils were isolated from the peripheral blood of healthy volunteers by a negative selection method. General purpose flow cytometry revealed that the purity of the isolated T lymphocytes, B lymphocytes, monocytes, neutrophils, and eosinophils were 95.8%, 88.4%, 57.6%, 97.8%, and 87.4%, respectively (**Fig. 1**). Subsequently, cells were measured using XE-2100 and XN-2000 analyzers, and their position on the differential leukocyte scattergrams, the XE-2100 DIFF scattergram (**Fig. 2A**), the XN-2000 WDF scattergram (**Fig. 2B**), and the XN-2000 WDF (SSC-FSC) scattergram (**Fig. 2C**) were examined and compared with those of whole peripheral blood.

All of the isolated leukocytes appeared in the same position when compared to whole peripheral blood. Moreover, the WDF scattergram showed a larger difference with respect to the intensity of side-scattered light between lymphocytes and monocytes than the DIFF scattergram, and it was confirmed that the cells were well separated.

2. The fluorescence intensity of each subtype of leukocytes after treatment with WDF-specific reagents

Each subtype of leukocyte isolated from the peripheral

blood of healthy volunteers was treated with the WDF-specific reagent and observed by CLSM (**Fig. 3**). As a result, we confirmed that mainly the cytoplasm of these cells were stained with the WDF-specific reagent. From the microscope images, we analyzed the intensity of fluorescent staining on a per-cell basis. When the fluorescence intensity of eosinophils was set at 1.00, the average fluorescence intensity values and standard deviations were 9.02 ± 0.49 for B lymphocytes, 9.81 ± 0.73 for T lymphocytes, 24.73 ± 2.29 for monocytes, and 2.11 ± 0.09 for neutrophils.

3. The morphology of each leukocyte subtype following treatment with each reagent

Each subtype of leukocyte isolated from the peripheral blood of healthy volunteers was treated with the WDF-specific or the DIFF-specific reagent under the same conditions in the XN or XE analyzers. Samples were then fixed, cut, electron-stained, and observed by TEM (**Fig. 4**). Both reagents disrupt the cell membranes of cells in the following order of severity: B lymphocytes, T lymphocytes, monocytes, neutrophils, and eosinophils. The WDF-specific reagent generally caused less damage to the cell membrane than the DIFF-specific reagent. The cell membrane of lymphocytes is significantly disrupted by both reagents, and almost all cytoplasm is lost. Although the damage to cell membranes is less with the WDF-specific reagent than with the DIFF-specific reagent, both reagents cause a similar loss of intracellular structures because lymphocytes have few organelles (**Fig. 4**: B lymphocytes, T lymphocytes). On the other hand, monocytes have many more organelles than

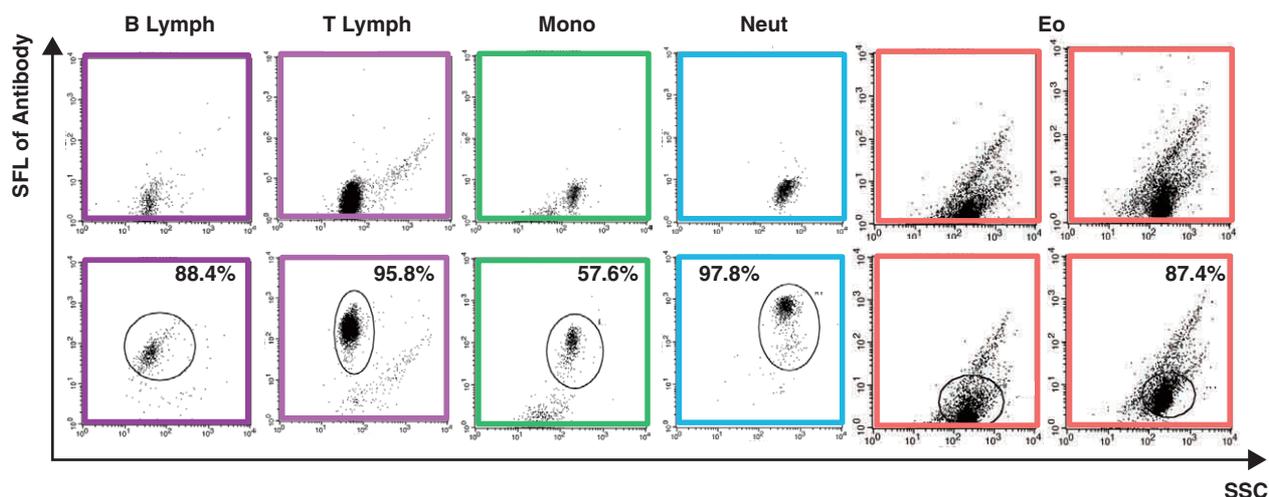


Fig. 1 Purity check of each subtype of leukocyte isolated from healthy volunteers

The vertical axis indicates staining intensity with a specific antibody for each leukocyte, and the horizontal axis is the SSC.

(Upper) Each subtype was stained and compared to the FITC-conjugated or PE-conjugated negative control via analysis with FACSCalibur.

(Lower) Each subtype of leukocyte was stained with a specific monoclonal antibody (T lymphocyte, anti-CD3 antibody; B lymphocytes, anti-CD19 antibody; monocytes, anti-CD14 antibody; neutrophils, anti-CD16 antibody; eosinophils, anti-CD123 antibody and anti-CD294 antibody).

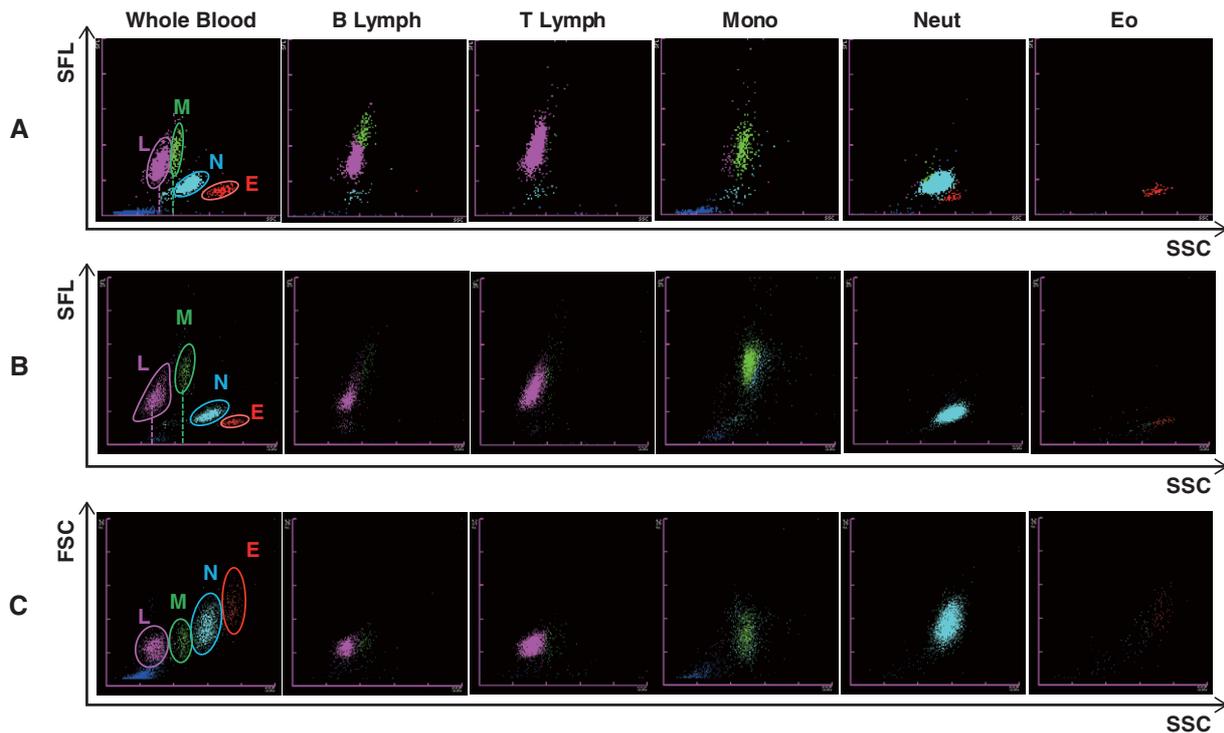


Fig. 2 The position of whole blood and each subtype of leukocytes isolated from healthy volunteers in the XE-Series and XN-Series
 (A) An XE-Series DIFF scattergram, where the vertical axis refers to the SFL and the horizontal axis refers to the SSC.
 (B) An XN-Series WDF scattergram, where the vertical axis refers to the SFL and the horizontal axis refers to the SSC.
 (C) An XN-Series WDF (FSC-SSC) scattergram, where the vertical axis refers to the FSC and the horizontal axis refers to the SSC.
 From the left: whole blood, B lymphocyte, T lymphocyte, monocyte, neutrophil, and eosinophil.

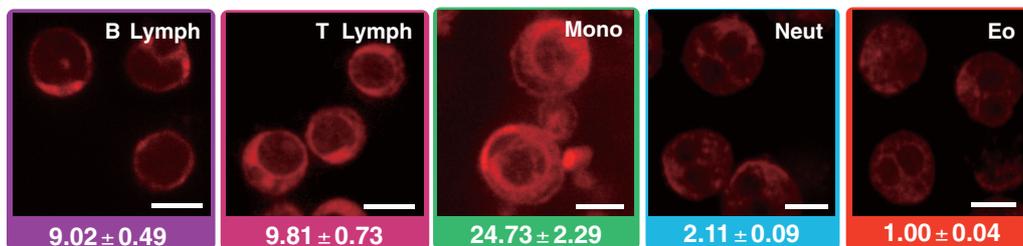


Fig. 3 Fluorescence intensities and images of each subtype of leukocyte stained with the WDF-specific reagent
 The CLSM image of each isolated subtype of leukocyte stained with the WDF-specific reagent. The number at the bottom of the panel indicates the mean fluorescence intensity and standard error, with the mean fluorescence intensity of T lymphocytes equal to 1.00.
 From the left: B lymphocyte, T lymphocyte, monocyte, neutrophil, and eosinophil.
 Bar = 5µm.

lymphocytes, and the degree of damage with both reagents reflects the complexity of intracellular structure; monocytes treated with the DIFF-specific reagent retain more of their intracellular structure than those treated with the WDF-specific reagent (**Fig. 4**: Monocytes). As for neutrophils, the DIFF-specific reagent affected them more than the WDF-specific reagent, which causes more cell membrane damage and leads to more cytoplasmic loss than with the WDF-specific reagent. For eosinophils, however, the DIFF-specific reagent maintained the intracellular structure more clearly than the WDF-

specific reagent, and both reagents maintain the characteristic large granules (**Fig. 4**: Eosinophils). Fixed samples were coated with osmium and observed by SEM for their surface structure and size (**Fig. 5**). This revealed that cell size increased from lymphocytes (smallest) to monocytes to neutrophils to eosinophils (largest). SEM analysis also confirmed that the cells treated with the DIFF-specific reagent had more cell membrane damage and were bulging than those treated with the WDF-specific reagent.

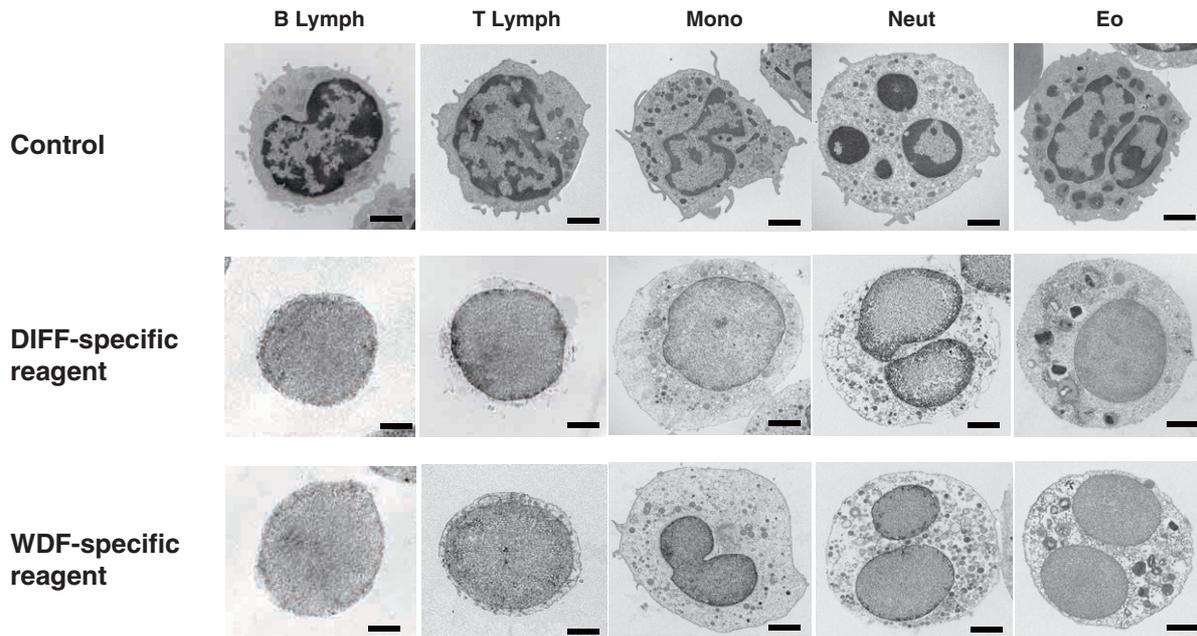


Fig. 4 TEM images of the isolated subtypes of leukocytes treated with each specific reagent (Upper) TEM images of the isolated subtypes of leukocytes before treatment with specific reagents. (Middle) TEM images of the isolated subtypes of leukocytes after treatment with the DIFF-specific reagent. (Lower) TEM images of the isolated subtypes of leukocytes after treatment with the WDF-specific reagent. From the left: B lymphocyte, T lymphocyte, monocyte, neutrophil, and eosinophil. Bar = 1 μ m.

4. The cross sectional area of each leukocyte subtype following treatment with WDF reagents

Fixed samples were observed by SEM to measure the cross sectional area of each leukocyte using a 3D laser microscope. The cross sectional area of each leukocyte increased from lymphocytes (smallest) to monocytes to neutrophils to eosinophils (largest), in that order (**Fig. 6**). As a result, it was confirmed that the sizes of the leukocytes treated with the WDF-specific reagent were, in order: eosinophils > neutrophils > monocytes > lymphocytes.

DISCUSSION

In this study, we used normal leukocytes from healthy volunteers to examine the principle of leukocyte differentiation and explore the reasons for the positional appearance of each leukocyte subtype on WDF scattergrams compared with DIFF scattergrams. First, the purity of each leukocyte subtype isolated from the peripheral blood of healthy volunteers was confirmed by

general flow cytometric analysis; the purity was over 85% for all subtypes except for the monocytes (**Fig. 1**). Isolated monocytes likely have a lower purity because platelets adhere to monocytes (**Fig. 3, 5**: Image of monocytes). In whole peripheral blood, it was confirmed that all leukocytes appeared correctly when both analyzers (XE-2100 and XN-2000) were used. On the other hand, among the isolated leukocytes, all leukocytes appeared at the correct position on the WDF scattergram, but some leukocytes were recognized as other blood cells in the DIFF scattergram. As a result, the XN-2000 analyzer can separate leukocyte subtypes more accurately than the XE-2100 analyzer. B- and T lymphocytes appeared in slightly different positions on the scattergrams. Classification of lymphocyte subtypes requires flow cytometric analysis using CD antibodies, but this data suggests it is possible that an abnormal ratio of lymphocyte subtypes can potentially be identified using automated hematology analyzers⁸⁾. Fluorescence staining with a WDF-specific reagent was observed by CLSM, and we found that it mainly stained the cytoplasm. It was thought that polymethine dye stained the nucleic acids in the cytoplasm. The staining intensity after treatment with the reagents was higher in

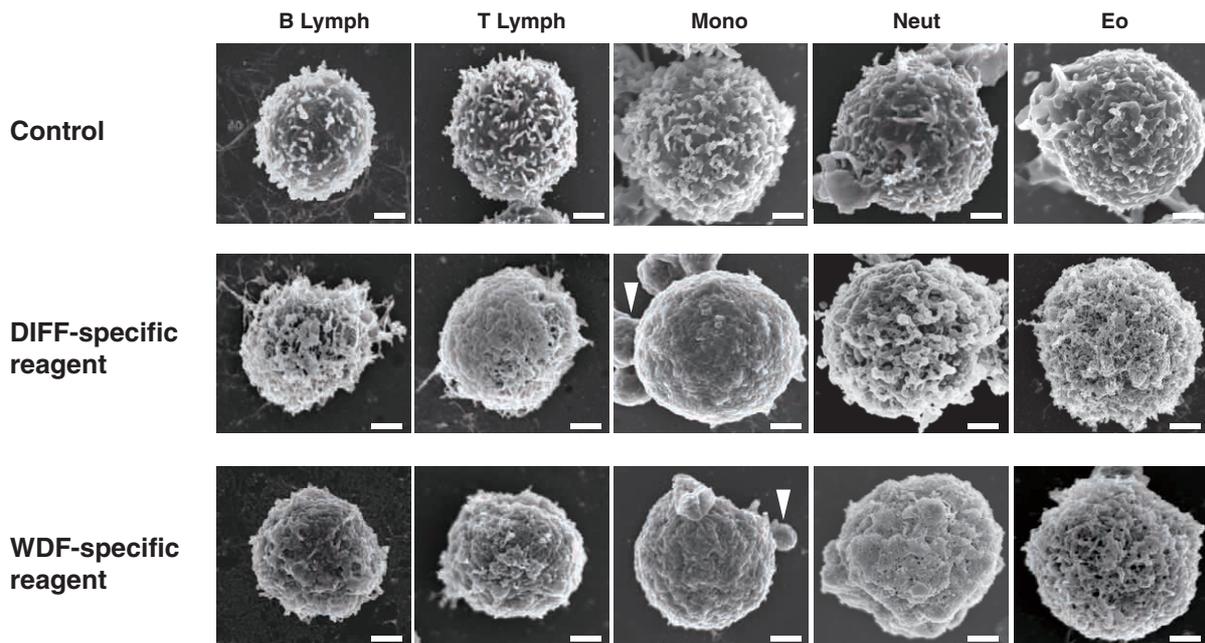


Fig. 5 SEM images of the isolated subtypes of leukocytes treated with each specific reagent
 (Upper) SEM images of the isolated subtypes of leukocytes before treatment with specific reagents.
 (Middle) SEM images of the isolated subtypes of leukocytes after treatment with the DIFF-specific reagent.
 (Lower) SEM images of the isolated subtypes of leukocytes after treatment with the WDF-specific reagent.
 From the left: B lymphocyte, T lymphocyte, monocyte (▼: platelet), neutrophil, and eosinophil.
 Bar = 1 μ m.

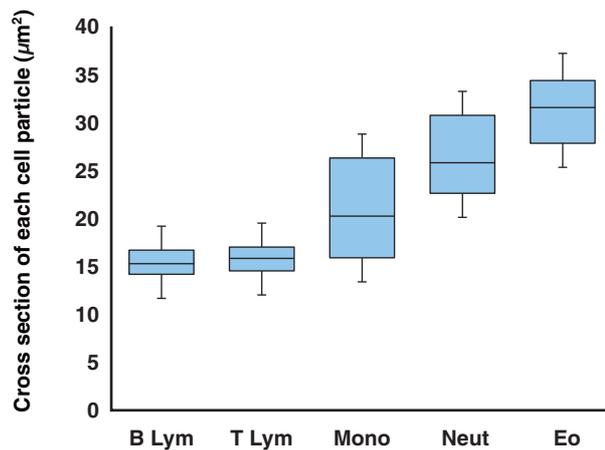


Fig. 6 Cross section of images of the isolated leukocyte subtypes treated with the WDF-specific reagent
 The cross section of each subtype of leukocyte treated with the WDF-specific reagent for SEM samples was measured with a 3D laser microscope.
 The vertical axis indicates the cross section of each subtype of leukocyte [μ m²]. At least 100 cells were measured for each type of leukocyte.
 The top and bottom of the bar indicate the maximum and minimum, the box is the range of 50% of the data, and the horizontal line in the box indicates the median.
 From the left: B lymphocyte, T lymphocyte, monocyte, neutrophil, and eosinophil.

monocytes, T lymphocytes, B lymphocytes, neutrophils, and eosinophils in the order, which correlate with the fluorescence intensity on the WDF scattergram (**Fig. 3**). Next, the intracellular structure of leukocytes was observed by TEM, and we found that of all the subtypes of leukocytes, the intracellular complexity after treatment with reagents was simplest in the lymphocytes, followed by the monocytes, neutrophils, and eosinophils, which correlated with the side-scatter intensity on both the WDF and WDF (FSC-SSC) scattergrams (**Fig. 4**). Additionally, the size and diameter of the leukocytes were observed by SEM and 3D laser microscopy, and we demonstrated that after treatment with reagents, the cross sectional area of the lymphocytes was the smallest, followed by that of monocytes, neutrophils, and eosinophils, which correlated with the forward-scatter intensity on the WDF (SSC-FSC) scattergram (**Fig. 5, 6**). These observations indicate that each leukocyte cluster is separated by differences in the number of residual organelles and the detergent tolerance of cell membranes, with respect to each cell type, because these differences cause differences in the fluorescence intensity, internal complexity, and size⁹⁾.

We morphologically assessed the reason why the WDF scattergram has greater separation of lymphocytes and monocytes in side-scattered light than the DIFF scattergram. TEM observation revealed that the detergent in the DIFF-specific reagent has a greater effect on intercellular structures than the WDF-specific reagent (**Fig. 4**). Additionally, SEM observation confirmed that the DIFF-specific reagent have a greater effect on cell membranes than the WDF-specific reagent (**Fig. 5**). Because lymphocytes have a small number of organelles and a high nucleus to cytoplasm ratio, the complexity of their intracellular structure was not very different when the cell membranes are disrupted (**Fig. 4, 5: Lymphocyte**). In contrast, monocytes have a large number of organelles and a low nucleus to cytoplasm ratio, and hence damage to the cell membrane is reflected in the number of remaining organelles and the complexity of the intracellular structure (**Fig. 4, 5: Monocytes**).

Because the WDF-specific reagent cause less damage to the cell membranes than the DIFF-specific reagents, monocytes are able to better maintain their intracellular structure, which leads to a larger difference in the intensity of SSC between monocytes and lymphocytes. Therefore, the WDF scattergram shows greater separation between the clusters of monocytes and lymphocytes than the DIFF scattergram.

In this study, fluorescent microscopy images, electron microscopy images, and particle analysis results corresponded to the intensity of SFL, SSC, and FSC on WDF scattergrams. In addition, we clarified why the WDF scattergram shows greater separation between the lymphocytes and monocytes than the DIFF scattergram.

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