

Original Article

Direct flowcytometric analysis of eosinophils using a whole blood staining technique

Yumiko Kamada,¹ Akira Tsuda,² Hiroyuki Kayaba,¹ Osamu Urayama,² Hajime Oyamada,¹ Tomoe Kuwasaki,¹ Kiumi Takasaki,¹ Norihiro Saito² and Junichi Chihara²

¹Central Clinical Laboratory and ²Department of Clinical Laboratory Medicine, Akita University School of Medicine, Akita, Japan

ABSTRACT

Background: Flowcytometric analysis of purified eosinophils requires several hours to accomplish due mainly to purification of cells; moreover, it requires more than 20 mL blood and expensive reagents. The aim of the present study was to develop a method of direct flowcytometric analysis for eosinophils using whole blood.

Methods: Peripheral blood obtained from five healthy individuals (mean age 42 years) and 10 patients with eosinophilia (mean age 40.3 years) were used for analysis. We stained antigens (CD9 or CD16) and fixed cells with parabenzoquinone (PBQ) or paraformaldehyde (PFA) after hemolysis followed by treatment with *N*-octyl- β -glucopyranoside (OG).

Results: On comparison of forward scatter with side scatter dot plots among samples treated with hemolysis alone, PBQ fixation and PFA fixation, PBQ fixation showed the best results in discriminating eosinophils from other leukocyte fractions on the cytogram. Following fixation and permeabilization of cells, EG2, a secretory form of eosinophil cationic protein, was stained as an intracellular antigen. Flowcytometric analysis for EG2 showed a high positivity rate only in the eosinophil fraction. There were no differences in EG2 positivity or mean fluorescence intensity (MFI) between heparinized and EDTA-treated blood. Comparison of samples treated with OG at 6.0 and

7.4 mg/mL showed that the latter had a higher MFI for EG2 without significant change in the positivity rate.

Conclusions: The findings show that intra- and extracellular properties of eosinophils can be analyzed with whole blood using PBQ fixation and OG treatment at a concentration of 7.4 mg/mL. Direct flowcytometric analysis of eosinophils saves time and requires only a small amount of blood, both of which are advantageous for patients and laboratory workers.

Key words: EG2, eosinophils, flow cytometry, whole blood.

INTRODUCTION

In allergic inflammatory diseases, eosinophils are thought to play an important role as the main effector cells. Changes in surface or intracellular expression of antigens related to eosinophils are attracting special interest because of their expected clinical importance as indicators of pathological conditions in allergic inflammatory diseases. So far, purified eosinophils have been mainly used for analysis of eosinophils.¹ Eosinophils are purified by negative depletion of CD3, CD14, CD16 and CD20,² using the magnetic bead method with high purity at good yields;³ however, time and skill are required to obtain eosinophils maintaining *in vivo* functions. The conventional method for analysis using purified eosinophils is not likely to be widely accepted by routine clinical laboratories because of the time involved, the expense and the amount of blood required for purification of eosinophils. Recently, Holden *et al.*⁴ reported a FOG method in which cells are fixed with paraformaldehyde (PFA) after hemolysis of whole blood followed by

Correspondence: Dr Junichi Chihara, Department of Clinical and Laboratory Medicine, Akita University School of Medicine, 1-1-1 Hondo, Akita 010-8543, Japan.

Email: chihara@hos.akita-u.ac.jp

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N-octyl- β -glucopyranoside (OG) treatment to increase membrane permeability for the staining of intracellular antigens. Krug *et al.*⁵ detected intracellular granular proteins of eosinophils using parabenzoquinone (PBQ) instead of PFA. Based on these reports and with an aim to develop a simple method for flowcytometric analysis of surface and intracellular expression of various molecules, such as adhesion molecules, chemokine receptors and granular proteins of eosinophils, in a routine clinical laboratory, techniques for whole blood flowcytometric analysis of eosinophils were evaluated. First, the purity of eosinophils gated on the cytogram in whole blood flowcytometry was determined by staining surface markers of eosinophils (CD16 negative, CD9 positive). Then, using an EG2 monoclonal antibody,⁶ a secretory type of eosinophil cationic protein (ECP) expressed intracellularly by eosinophils, EG2 was also detected and investigated to determine whether intracellular antigens can be evaluated as well as surface antigens using the whole blood flowcytometry technique.

METHODS

There were 15 participants in the present study, comprising five healthy individuals (age range 37–51 years; mean age 42 years) and 10 patients with eosinophilia, including four patients with eosinophilic pneumonia (age range 45–65 years; mean age 42 years), three patients with bronchial asthma (age range 23–56 years; mean age 40 years) and three patients with atopic dermatitis (age range 18–26 years; mean age 22 years). Eosinophilia was defined as more than 700 eosinophils/ μ L in peripheral blood.

Surface staining for whole blood flowcytometry

For the identification of eosinophils, 10 μ L phycoerytherin (PE)-labeled anti-CD9 monoclonal antibody (clone M-L13; Pharmingen, San Diego, CA, USA) or PE (or fluorescein isothiocyanate (FITC) in double color staining)-labeled anti-CD16 monoclonal antibody (clone 3G8; Pharmingen) was added to 100 μ L heparinized or EDTA-treated blood and incubated. Unless otherwise mentioned, the incubation was performed at 4°C for 30 min. Then, 2 mL lysing solution (Beckton Dickinson, Franklin Lakes, NJ, USA) was added and incubated for 10 min at room temperature in the dark for hemolysis. After centrifugation at 300 g for 5 min, the supernatant was removed and 100 μ L of 0.4% PBQ

(Wako Pure Chemicals, Tokyo, Japan) or 4% PFA (Wako Pure Chemicals) was added to the remaining cells and the mixture was kept at room temperature in the dark for 10 min to fix the cells. After washing once with phosphate-buffered saline (PBS), cells were reacted with 100 μ L OG (Wako Pure Chemicals) at two final concentrations of 6.0 or 7.4 mg/mL to increase membrane permeability. After incubation for 5 min at room temperature in the dark, cells were washed twice with PBS and resuspended in 400 μ L PBS for flowcytometric analysis.

For the detection of various proteins other than CD9 and CD16 expressed on the surface of eosinophils, monoclonal antibodies against the proteins targeted by the study were used. Fluorescein isothiocyanate- or PE-labeled secondary antibodies were used when non-labeled primary antibodies were used. After incubation with the antibodies, cells were treated with lysing solution, PBQ and OG (7.4 mg/mL) in the same manner as described above. As an example of the surface staining of eosinophils in whole blood, the expression of Mac-1 on eosinophils is shown in the Results section.

Intracellular staining for whole blood flowcytometry

After treatment with lysing solution, PBQ and OG as described above, 5 μ L EG2 monoclonal antibody (Cabi-Pharmacia, Uppsala, Sweden) or irrelevant mouse IgG1 (DAKO, Kyoto, Japan) as an isotype control was added and incubated. After washing once with PBS, 100 μ L of 100-fold diluted FITC-conjugated goat antimouse Immunoglobulins (Biosource International, Camarillo, CA, USA) was added to the reaction mixture and incubated. Then, cells were washed once with PBS and resuspended in 500 μ L PBS for flowcytometry.

Flowcytometric analysis

More than 15 000 cells were counted for flowcytometric analysis by Facscan with LYSIS II software (Becton Dickinson). The positivity rate for the control was adjusted to less than 1%. The change in mean fluorescence intensity (Δ MFI), which was obtained by subtracting the control MFI from that of the sample, was used as an index of expression level. Because eosinophils have stronger spontaneous fluorescence than other leukocytes, adjustment of compensation levels was carefully performed between FL1 (FITC) and FL2 (PE) to avoid leakage of FITC and PE fluorescence into the detectors when cells were stained with double color. For the purpose of confirming

the constant condition of the cell analyzing system, Calib RATE beads (Becton Dickinson) were used for each measurement. Reproducibility of photomultiplier (PMT) voltage, adjustment for fluorescent dyes and detection sensitivity under the operating conditions were confirmed at each measurement.

Statistical analysis

All values are presented as the mean \pm SD. For analysis of the significance of differences between two groups, Student's *t*-test was used. In comparisons of three or more groups, Fisher's protected least significant difference (PLSD) method was used as a post hoc test, regarding $P < 0.05$ as significant after analysis of variance.

RESULTS

Comparison of cytograms of samples treated with hemolyzation alone, 4% PFA and OG or 0.4% PBQ and OG is shown in Fig. 1. In Fig. 1, the x and y axes represent the forward light scattering and side light scattering at 90°, respectively. By hemolyzation alone, neutrophil and eosinophil fractions overlapped each other and gating for eosinophils was difficult due to the unclear boundary. The boundary became clear after PFA and OG treatment; however, there was still a narrow overlapping area. Of the procedures tested, PBQ and OG treatment yielded the best cytogram for the gating of eosinophils (Fig. 1). The CD9-positivity rate in the gated area representing the eosinophil fraction was 91.9 ± 3.8 , 93.5 ± 2.15 and $95.4 \pm 1.7\%$ in samples treated with hemolyzation alone, PFA + OG and PBQ + OG, respectively (Fig. 2). Samples treated with PBQ and OG showed a lower CD16 positivity rate ($6.9 \pm 1.9\%$) in the gated area than samples treated with hemolyzation alone ($19.2 \pm 5.6\%$; $P < 0.05$) or PFA fixation ($9.6 \pm 3.1\%$; NS; Fig. 3). Based on these results, the intracellular EG2-positivity rate and MFI were evaluated in samples obtained from patients with eosinophilia after PBQ and OG treatment. Blood was sampled with two sample tubes, one containing heparin and the other containing EDTA as an anticoagulant. As in the reports of Hallden *et al.*,^{4,7} two different concentrations of OG (6.0 and 7.4 mg/mL) were used in the preparation of blood samples for comparison in the present study. The eosinophils showed a high positivity rate for EG2 regardless of the anticoagulants or OG concentrations (Fig. 4). Comparison of samples treated with OG at concentrations of

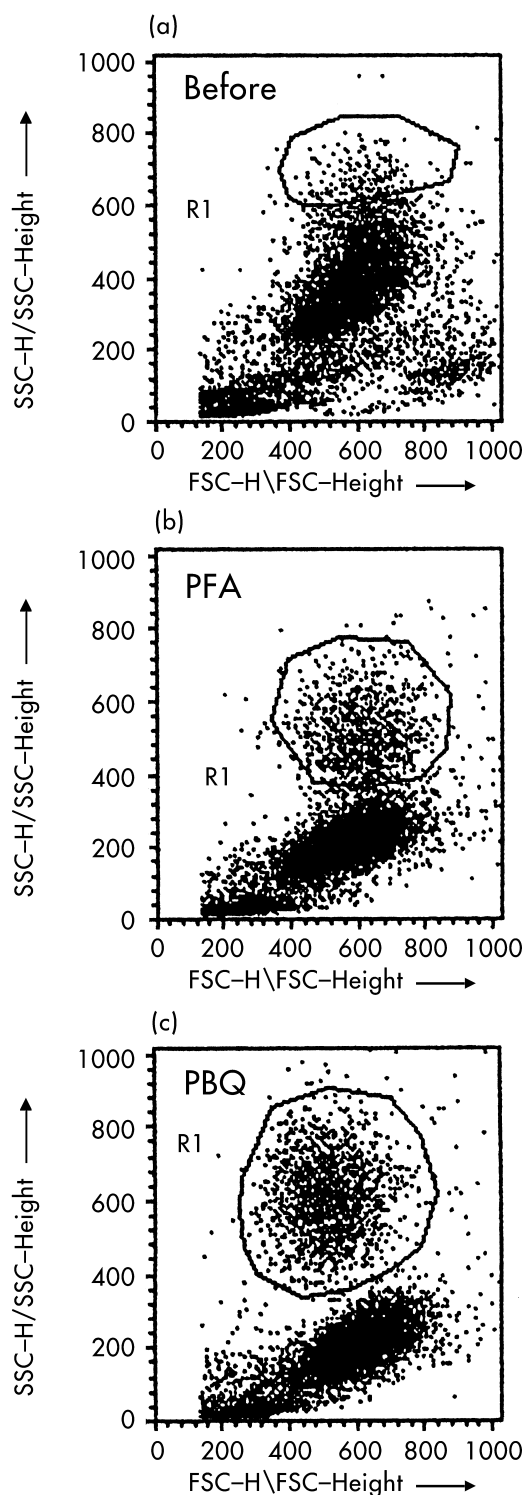


Fig. 1 Changes in cytograms of leukocyte fractions in whole blood after treatments. (c) The cytogram of peripheral whole blood treated with parabenzoquinone and *N*-octyl- β -glucopyranoside (OG) showed highly selective acquisition of eosinophils compared with other cytograms treated with paraformaldehyde and OG (b) or without treatment (a).

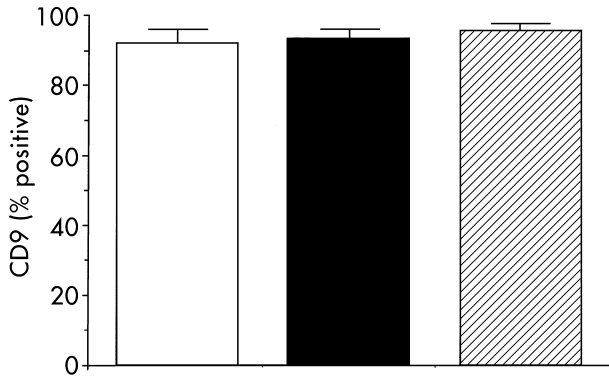


Fig. 2 CD9-positivity rate in the eosinophil gating field. The CD9-positivity rate was 91.9 ± 3.8 , 93.5 ± 2.15 and $95.4 \pm 1.7\%$ in samples treated with hemolyzation alone (□), paraformaldehyde + *N*-octyl- β -glucopyranoside (OG; ■) and parabenzoquinone + OG (▨), respectively.

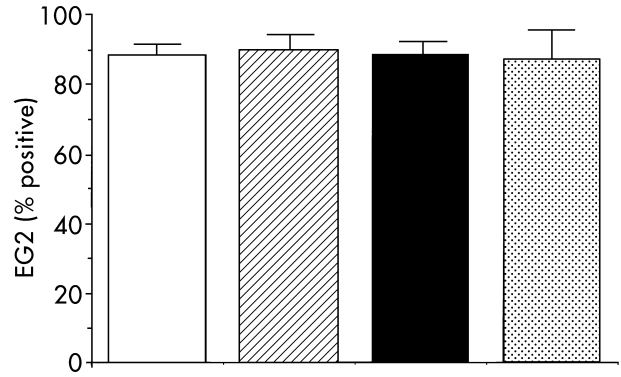


Fig. 4 The EG2 positivity rate in the eosinophil gating field. Eosinophil fractions of allergic patients were gated and examined for intracellular EG2-positivity rate using parabenzoquinone (PBQ) fixation. The eosinophils showed a high positivity rate for EG2 regardless of the anticoagulants or *N*-octyl- β -glucopyranoside (OG) concentrations used. The positivity rates for EG2 in the heparinized blood samples fixed with PBQ and permeabilized by OG at concentrations of 6.0 (□) and 7.4 mg/mL (▨) were 88.0 ± 3.2 and $88.9 \pm 4.8\%$, respectively. Samples treated with EDTA showed a positivity rate of 88.3 ± 3.4 and $86.9 \pm 8.3\%$ at OG concentrations of 6.0 (■) and 7.4 mg/mL (▩), respectively.

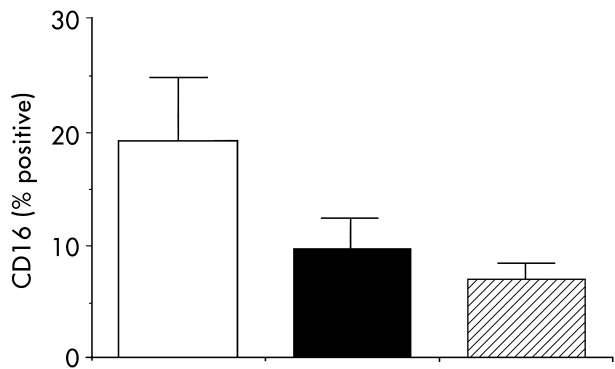


Fig. 3 CD16-positivity rate in eosinophil gating field. The sample treated with parabenzoquinone and *N*-octyl- β -glucopyranoside (OG; ▨) showed a lower CD16 positivity rate ($6.9 \pm 1.9\%$) in the gated area than samples treated with hemolyzation alone (□; $19.2 \pm 5.6\%$; $P < 0.05$) or paraformaldehyde fixation (■; $9.6 \pm 3.1\%$; NS).

either 6.0 or 7.4 mg/mL, the latter had a significantly higher MFI for EG2 than the former, regardless of the anticoagulant (Fig. 5). The positivity rates for EG2 in heparinized blood samples fixed with PBQ and permeabilized by OG at concentrations of 6.0 and 7.4 mg/mL were 88.0 ± 3.2 and $88.9 \pm 4.8\%$, respectively. Samples treated with EDTA showed a positivity rate of 88.3 ± 3.4 and $86.9 \pm 8.3\%$ at OG concentrations of 6.0 and 7.4 mg/mL, respectively. The MFI for EG2 in heparinized blood samples fixed with PBQ and permeabilized by

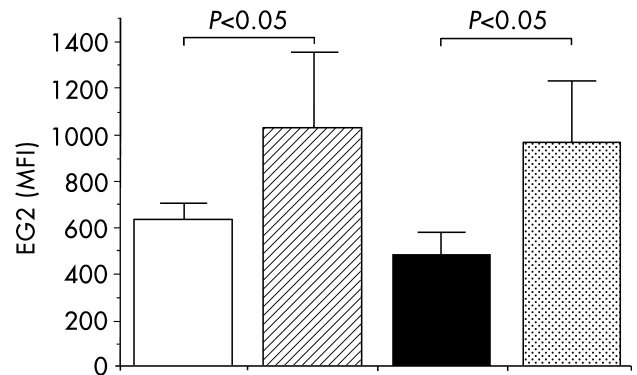


Fig. 5 Mean fluorescence intensity (MFI) for EG2 in the eosinophil gating field. Comparison of samples treated with *N*-octyl- β -glucopyranoside (OG) at 6.0 and 7.4 mg/mL showed that the latter had a significantly higher MFI for EG2 than the former, regardless of the anticoagulant. The MFI for EG2 in heparinized blood samples fixed with parabenzoquinone (PBQ) and permeabilized by OG at concentrations of 6.0 (□) and 7.4 mg/mL (▨) was 625.2 ± 72.3 and 1023.1 ± 325.8 FI, respectively. Samples treated with EDTA showed an MFI of 475.2 ± 101.6 and 960.2 ± 264.3 FI at OG concentrations of 6.0 (■) and 7.4 mg/mL (▩), respectively.

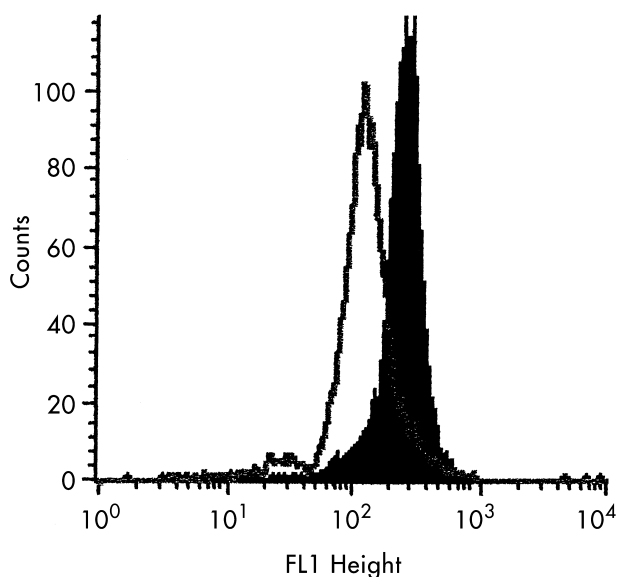


Fig. 6 Expression of Mac-1 on eosinophils. An example of whole blood flowcytometric analysis on the surface expression of Mac-1 on eosinophils is shown as histograms obtained from a patient with eosinophilia (■) and a normal subject (□). An increase in Mac-1 expression was demonstrated in the patient with eosinophilia compared with the normal subject.

OG at concentrations of 6.0 and 7.4 mg/mL were 625.2 ± 72.3 and 1023.1 ± 325.8 FI, respectively. Samples treated with EDTA showed a MFI of 475.2 ± 101.6 and 960.2 ± 264.3 FI at OG concentrations of 6.0 and 7.4 mg/mL, respectively.

An example of whole blood flowcytometric analysis on the surface expression of Mac-1 on eosinophils is shown in Fig. 6. An increase in Mac-1 expression in a patient with eosinophilia compared with that of a normal subject was demonstrated.

DISCUSSION

Although various methods for the flowcytometric detection of eosinophils in whole blood using the eosinophil property of strong spontaneous fluorescence have been investigated,^{8,9} these methods are not generally used because of the fact that the fluorescence of neutrophils increases in some pathological conditions.¹⁰ By staining cell surface CD9 and CD16 in whole blood and hemolyzing, followed by treatment with PBQ and OG, the

eosinophil fraction (CD9 positive, CD16 negative) was more easily gated on the cytogram separately from other leukocyte fractions. In the gated area, CD9 was positive in $95.4 \pm 1.7\%$ of cells and CD16, representing neutrophil contamination, was positive in $6.9 \pm 1.9\%$ of cells, which was a good result. The fact that the cytogram, with an unclear boundary between eosinophils and neutrophils, has a high CD16 positivity rate may suggest that the neutrophils (CD16 positive) are the main contaminating cells of the eosinophil fraction.

The major component of the lysing solution used in the present study was 5.0% diethyleneglycol and 1.5% formaldehyde, and formaldehyde may fix the cells at hemolization to some extent. Carulli *et al.*⁹ reported that a cytogram of the eosinophil fraction could be obtained by treatment with lysing solution alone and the percentage of eosinophils in the gated area showed a good correlation with the results obtained by an automatic blood cell counter. However, in the present study, when whole blood was treated with lysing solution alone, the eosinophil fraction and neutrophil fraction overlapped each other, making the gating of the eosinophil fraction difficult. On gating a high granularity area of the leukocyte fraction in the sample treated with lysing solution alone, 19.2% of gated cells were CD16 positive, showing contamination by neutrophils. Using a hypotonic salt solution as a hemolytic agent, the eosinophils lessened their homogeneity on their size and granularity, resulting in a scattered forward scatter compared with side scatter dot plot and unclear boundaries between other leukocyte fractions (data not shown). Krug *et al.*⁵ compared PBQ and PFA fixation and noted that non-specific binding of FITC occurred in samples fixed by PFA, whereas clear separation of the eosinophil fraction from other leukocyte fractions was obtained by PBQ fixation. Although we did not confirm non-specific binding of FITC in samples fixed with PFA, we did obtain consistent results in that the eosinophil fraction was clearly gated using PBQ fixation. *N*-Octyl- β -glucopyranoside, which was used to increase membrane permeability, is a non-ionic surfactant, and the critical micelle concentration is reported to be 0.74%.⁴ Hallden *et al.*⁷ stained cytoplasmic antigens in lymphocytes with antivimentin and determined that the optimal OG concentration lies within a range from 6.0 to 8.0 mg/mL. Later, in 1993, Hallden and Hed⁴ reported the FOG method, which is a method of staining eosinophil intracellular granular protein, setting the OG concentration to 0.74%. In the present study, intracellular staining was performed at two different OG concentrations

(6.0 and 7.4 mg/mL). As a result, the EG2-positivity rate did not differ significantly between the two OG concentrations, but MFI was higher in cells treated with 7.4 mg/mL of OG, representing increased cell membrane permeability. Furthermore, although the data are not shown, the MFI for EG2 was significantly higher in asthmatic patients than in healthy individuals. The significance of the difference in MFI between asthmatic patients and healthy individuals was greater in samples permeabilized using OG at 7.4 mg/mL than in samples permeabilized using OG at 6.0 mg/mL,¹¹ indicating that 7.4 mg/mL OG is appropriate for EG2 measurements. In the present study, heparin and EDTA were used as anticoagulants. Because there was no significant difference in either the EG2-positivity rate or MFI between heparinized and EDTA-treated blood, when measurement of intracellular EG2 antigens is incorporated into a routine test as an index of activated eosinophils,^{12,13} an appropriate anticoagulant for individual patients can be selected. Sano *et al.*¹⁴ reported a method for whole blood flow-cytometric analysis of eosinophils using a double-staining technique, which distinguishes very late antigen (VLA)-4-positive eosinophils from VLA-4-negative neutrophils.¹⁴ Compared with the method of Sano *et al.*, our method has some advantages, as described below. First, our method does not require an expensive antibody only for the detection of eosinophils. Second, in the method of Sano *et al.*, one must be aware of the existence of anti-VLA-4 antibody bound to VLA-4 on the eosinophils when selecting a secondary antibody to detect the other proteins to be evaluated. Third, the method of Sano *et al.* requires calibration for double staining even if the evaluation could be performed with single-color staining by our method.

Our method allows intra- and extracellular analysis of eosinophil properties using whole blood without an eosinophil separation procedure that consumes several hours. Moreover, only 100 μ L blood is required for each sample tube using our method, which is a great advantage, especially for pediatric patients. This method brings us one step closer to finding a simple, non-time consuming test for the evaluation of allergic diseases through eosinophils.

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