

Clinical utility of a passive immune basophil activation test for the analysis of allergic transfusion reactions

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BACKGROUND: In previous studies, we demonstrated that the basophil activation test, which is performed using patient blood and the supernatants from transfused blood components, was able to elucidate not only the causative relationship between allergic transfusion reactions and the transfusion but also the mechanisms behind allergic transfusion reactions. However, for a large number of allergic transfusion reactions, patients are in a state of myelosuppression, and the basophil activation test cannot be performed for these patients because there are insufficient numbers of peripheral blood basophils.

STUDY DESIGN AND METHODS: To overcome this obstacle, we developed a passive immune basophil activation test, in which patient plasma and residually transfused blood are used as the patient's sources of immunoglobulin E and allergen, respectively, whereas healthy volunteer basophils serve as the responder cell source. The passive immune basophil activation test was performed for two patients who had severe allergic transfusion reactions, using supernatants of the residual platelet concentrates and the patients' own immunoglobulin E.

RESULTS: There were no differences in either surface immunoglobulin E or activation in response to allergens between untreated basophils and so-called quasi-basophils, in which immunoglobulin E was replaced by a third party's immunoglobulin E. In these patients, the supernatants of the residual platelet concentrates exclusively activated basophils in response to quasi-basophils onto which the patients' immunoglobulin E, but not a third party's immunoglobulin E, was bound.

CONCLUSION: The passive immune basophil activation test may help clarify the causal relationship between allergic transfusion reactions and transfused blood, even when patients experience myelosuppression.

Allergic transfusion reactions (ATRs) are the most common adverse events associated with the transfusion of plasma, and particularly the transfusion of platelet concentrates (PCs).¹⁻⁵ It is believed that ATRs are mediated by mast cells and/or basophils. In general, the activation of mast cells or basophils and subsequent histamine release are mediated by two pathways: the allergen/immunoglobulin E (IgE)-dependent and allergen/IgE-independent pathways.^{6,7} However, the mechanisms by which ATRs occur in the majority of patients remain unknown, likely because there is no standard assay system in which to explore these mechanisms. The tryptase test may be used,⁷⁻⁹ but it often cannot confirm the causative relationship between the reaction and the transfusion,⁹⁻¹¹ much less the mechanisms behind ATRs.

To overcome this obstacle, several investigators have applied the basophil activation test (BAT) to transfusion medicine. Dewachter and colleagues and Nubret and coworkers independently reported several cases of immediate allergic hypersensitivity to the transfusion of methylene

ABBREVIATIONS: AHlgE = anti-human IgE; ATR(s) = allergic transfusion reaction(s); BAT = basophil activation test; BRM(s) = biologic response modifier(s); FCM = flow cytometry; pi-BAT = passive immune basophil activation test; RT = room temperature; SN(s) = supernatant(s).

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TABLE 1. Patient characteristics

Characteristic	Case 1	Case 2
Age	53 years*	54 years
Sex	Male*	Female
Disease	Acute lymphoblastic leukemia*	Acute mixed leukemia
Treatment status	Day 19 of unrelated bone marrow transplantation*	Remission after chemotherapy
Time of anaphylaxis after starting transfusion	30 minutes*	15 minutes
Symptoms	Urticaria, angioedema, and dyspnea*	Urticaria, angioedema, and dyspnea
No. of previous transfusions (platelet and plasma)	>20	14
Adverse reactions observed during previous transfusion	None	1 Minor rash after PC transfusion

* These data are derived from our previous report.¹⁶

blue-treated plasma, revealing that all patients experienced methylene blue-induced, IgE-mediated anaphylaxis using patient blood and residually transfused plasma for the BAT.^{12,13} We demonstrated that supernatants (SNs) in residually transfused blood from patients with ATRs can activate basophils derived from the whole blood of healthy volunteers; this activation was elicited through an allergen/IgE-dependent pathway, which could be blocked by the addition of dasatinib, an inhibitor of IgE-mediated basophil activation.^{14,15} Subsequently, we performed the BAT for two patients with severe ATRs using the SNs from the residual PCs (PC-SNs) and the patients' own basophils; the results demonstrated a causal relationship between the reactions and the transfusion. Basophil activation appeared to be allergen-IgE-dependent, although the specific allergens could not be identified.¹⁶

However, considering that approximately 70% of patients with ATR who receive transfusion with PCs contract a hematologic malignancy, aplastic anemia, or solid carcinoma, it is highly probable that treatment of the primary diseases causes myelosuppression, making it difficult to obtain a sufficient number of basophils for the BAT in such patients. Indeed, both patients described here experienced myelosuppression; consequently, the BAT was performed 1 month and 2 weeks after the ATRs, respectively. Therefore, the difficult collection of basophils from patients who experience myelosuppression is a major limitation of the BAT.

Ishizaka and his colleagues conducted a study in which monkey lung fragments were passively sensitized with IgE antibodies from human serum; the release of histamines and leukotrienes was assessed upon challenge with either a specific allergen or an anti-IgE antibody.¹⁷ Pruzansky and coworkers reproduced the passive sensitization of basophils with IgE in vitro and then applied this technology to the field of allergic diseases.¹⁸ These authors demonstrated that basophils from healthy volunteers, whose own receptor-bound IgE was replaced by patient IgE, were activated by allergens to which the patients were sensitized. We call this system the "passive immune" BAT (pi-BAT).

In this report, we investigated whether the pi-BAT can be applied to the field of transfusion medicine to

assess the causal relationship between ATRs and the transfusion, especially in patients with myelosuppression.

MATERIALS AND METHODS

Patients

Two patients with transfusion-related anaphylaxis, who presented in December 2014 at the Shizuoka Cancer Center (Case 1) and March 2016 at the Kansai Medical University Medical Center (Case 2), were included in this study (Table 1). Case 1 was a 53-year-old man with acute lymphoblastic leukemia who underwent allogeneic bone marrow transplantation from an unrelated donor with a one-antigen mismatch at molecular complete remission. Nineteen days after transplantation, he experienced anaphylaxis while receiving a leukoreduced, ABO-identical PC transfusion. The results of a regular BAT performed on this patient have already been reported.¹⁶ Case 2 was a 54-year-old woman with acute mixed leukemia who experienced anaphylaxis while also receiving leukoreduced, ABO-identical PCs.

PCs associated with ATRs

All PCs in this study, both leukoreduced and irradiated, were prepared from single donors using apheresis. The residual PC products, which induced an ATR in Case 1, were collected immediately at the hospital after the occurrence of the ATR. Cell-free PC-SNs were separated by centrifugation at $3000 \times g$ for 30 minutes at 4°C and stored at -40°C.¹⁵ In Case 2, residual PCs were stored at the hospital for 2 days at 4°C. PC-SNs were prepared immediately after the arrival of the blood bags at our blood center and were stored at -40°C. This study was approved by the Institutional Review Board of the Japanese Red Cross Society Headquarters (no. 2015-48).

Preparation of peripheral whole blood

Ethylene diamine tetraacetic acid (EDTA) was added to the peripheral whole blood collected from the patients. In addition, EDTA-treated whole blood was collected from healthy laboratory personnel at our blood center who had been identified as either lacking or possessing specific IgE

against inhalants and food allergens, as described below. Plasma samples were separated from both patients and healthy laboratory personnel by centrifugation at $3000 \times g$ for 10 minutes at 4°C and were used as an IgE source from patients or healthy personnel with or without allergies to inhalants and/or food. To separate mononuclear cells from the whole blood of healthy laboratory personnel, 6% (weight/volume) hydroxyethyl starch 40 (HES40) was added at a final concentration of 1.2%. A mononuclear cell-rich SN was then separated by centrifugation at $700 \times g$ for 5 minutes at 4°C to enhance the red blood cell sedimentation rate. Peripheral blood mononuclear cells (PBMCs) were finally isolated from the mononuclear cell-rich SN by centrifugation at $1200 \times g$ for 5 minutes at 4°C . The PBMCs were used as the source of untreated or treated basophils.

Reagents

Purified mouse anti-human IgE (AHIgE) monoclonal antibody (MoAb) was obtained from Beckman Coulter. Fluorescein isothiocyanate-conjugated mouse anti-HLA-DR (human leukocyte antigen-antigen D related) and phycoerythrin (PE)-conjugated mouse anti-CD203c MoAbs were also purchased from Beckman Coulter. PE-cyanine 5 (Cy5)-conjugated mouse anti-CD123 MoAb and fluorescence-activated cell-sorting (FACS) lysis solution were purchased from BD Biosciences. PE-conjugated mouse AHIgE MoAb was purchased from BioLegend. Pollen from the Japanese cedar and house dust extract were purchased from Torii Pharmaceutical. *N*-Formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) and lipopolysaccharide (LPS) were obtained from Sigma-Aldrich. Recombinant human interleukin 3 (IL-3) and recombinant human interleukin 33 (rhIL-33) were purchased from AbD Serotec and R&D Systems, respectively. Dasatinib was obtained from Trout Research Chemicals. Dimethyl sulfoxide (DMSO) was obtained from Thermo Fisher Scientific Inc. HES40 was purchased from NIPRO. Heparin sodium and CaCl_2 were purchased from Mochida Pharmaceutical Company, Ltd., and Nacalai Tesque, respectively. We used three third-party plasmas that contained no specific IgE against house dust or Japanese cedar pollen (Type A), house dust-specific IgE (Type B), or Japanese cedar pollen plus house dust-specific IgE (Type C) and determined the concentration of specific IgEs against Japanese cedar pollen and house dust using PhadiaImmunoCap. The concentrations of IgE against Japanese cedar pollen in the Type A, B, and C plasmas were <0.34 , <0.34 , and 4.01 UA/mL , respectively, and the concentrations of house dust were <0.34 , 5.45 , and 6.11 UA/mL , respectively.

The classic BAT

Aliquots of whole blood ($100 \mu\text{L}$), which were collected from patients and healthy laboratory personnel (controls), were incubated with $5 \mu\text{L}$ PC-SNs at 37°C for 30 minutes. As

positive controls, the basophil agonists AHIgE MoAb and rhIL-3 were used in place of PC-SNs at final concentrations of 0.5 and $1 \mu\text{g/mL}$, respectively. After incubation, the mixtures were washed with phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and 10 mM EDTA (PBS/BSA/EDTA). The extent of basophil CD203c expression was analyzed by flow cytometry (FCM) (FACS-Canto II; BD Biosciences), as described below. To inhibit IgE/IgE receptor-triggered basophil activation, the whole blood was preincubated in the presence or absence of dasatinib ($3 \mu\text{M}$) at 37°C for 30 minutes and then incubated with PC-SNs at 37°C for an additional 30 minutes.^{15,16} Dasatinib was first dissolved in a small amount of DMSO and then diluted with PBS. DMSO was used to dilute dasatinib and thus was used as a negative control for dasatinib. When dasatinib was used in the BAT assay, the final dilution of DMSO was calculated as 1:13,300. DMSO was similarly diluted in the BAT assay when used as a negative control.

Establishment of basophils after replacement of receptor-bound IgE

Receptor-bound IgE from the responder cell source of basophils was replaced by autologous or third-party IgE using the method described by Santos and coworkers with minor modifications.¹⁹ Briefly, HES40, instead of histopaque-1077, was used to separate PBMCs, according to a standard method.^{20,21} To remove receptor-bound IgE, 5×10^6 PBMCs were suspended in 1 mL lactic acid solution (13.4 mM lactate, 140 mM NaCl, and 5 mM KCl) and incubated for 5 minutes on ice. To stop the reaction, the PBMC suspension was neutralized by the addition of 1 mL human serum albumin solution (RPMI-1640 medium, pH 8.0; supplemented with 0.5% human serum albumin and 12 mM Tris-HCl). The cells were centrifuged at $1200 \times g$ for 5 minutes at 4°C . After washing with PBS, the cell pellet was resuspended in 0.5 mL plasma supplemented with 10 U/mL heparin sodium and 10 mM CaCl_2 and incubated for 60 minutes at 37°C . The plasma used was derived from the same healthy laboratory personnel, other personnel with or without the specific IgE to inhalants and/or food allergens, or the patients with ATRs. We defined basophils in the cell pellet, in which their own receptor-bound IgE was replaced by third-party IgE, as “quasi-basophils.” The origin of receptor-bound IgE on the quasi-basophils is described in parentheses for the sake of convenience, for example, “quasi-basophils (IgE, Patient 1).”

The pi-BAT

Aliquots of 2.5×10^5 PBMCs, which contained either untreated basophils or quasi-basophils, were incubated with $5 \mu\text{L}$ PC-SNs at 37°C for 30 minutes. These mixtures were then washed twice with PBS/BSA/EDTA. The extent of basophil CD203c expression was analyzed by FCM, as described below. In some experiments, PBMCs containing

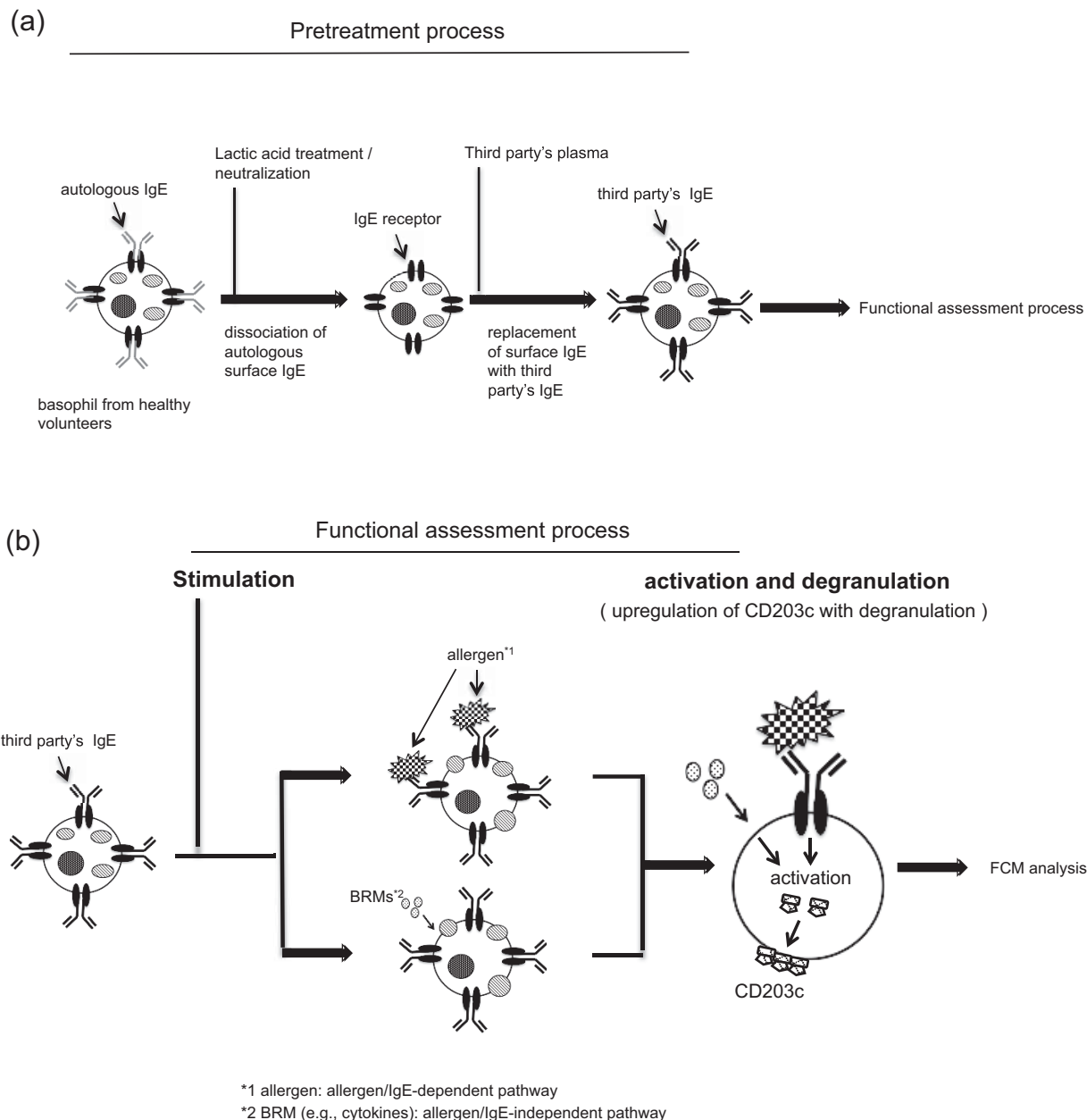


Fig. 1. The passive immune basophil activation test process. The pi-BAT is configured into two processes: (a) the pretreatment process and (b) the functional assessment process. In the pretreatment process, IgE of basophils' own origin was replaced with that of third-party origin by lactic acid treatment and a subsequent treatment with third-party plasma. In the functional assessment process, the activation of basophils is mediated by two pathways: the allergen/IgE-dependent and allergen/IgE-independent pathways. The allergen/IgE-dependent pathway occurs after high-affinity FcεRI (the high-affinity receptor for the Fc region of immunoglobulin E) aggregates in response to the cell surface binding of IgE in complexes with specific allergens. It has been speculated that the basophil activation in the alternative allergen/IgE-independent pathway occurs through biologic response modifiers, such as accumulated inflammatory cytokines, chemokines, and damage-associated molecular patterns, in stored blood components.

quasi-basophils were incubated with several concentrations of stimulatory agents, such as AHlgE MoAb, fMLP, LPS, rhIL-3, or rhIL-33 (basophil agonists that function in an IgE/IgE receptor-independent manner), at 37°C for 30 minutes.

Flow cytometry

After basophil activation, as described above, the samples were incubated at 4°C for 15 minutes with 10 µL fluorescein isothiocyanate-conjugated anti-HLA-DR MoAb, PE-conjugated anti-CD203c MoAb, and PE-Cy5-conjugated

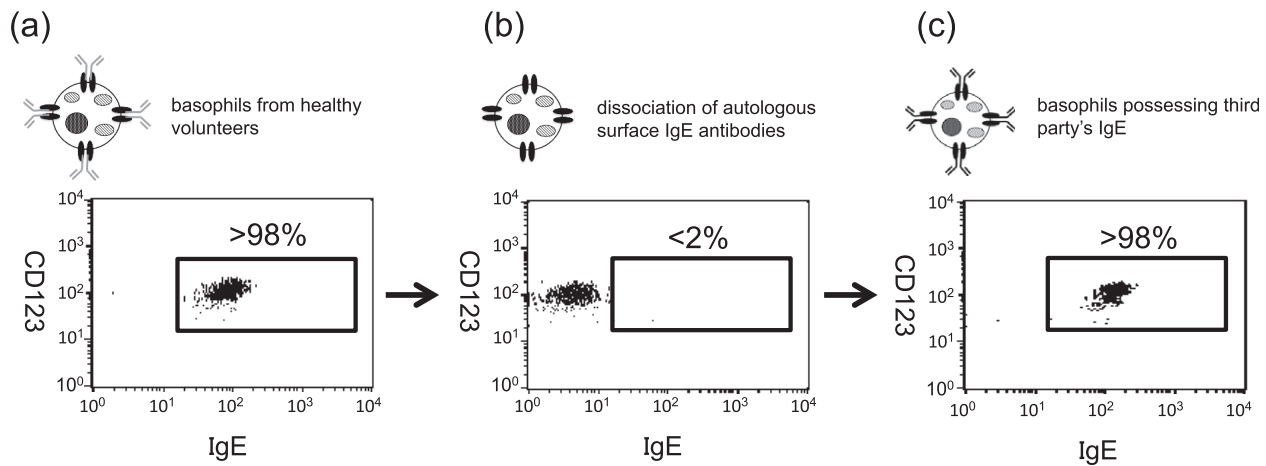


Fig. 2. Replacement of receptor-bound IgE after treatment with lactic acid and third-party plasma. Peripheral blood mononuclear cell samples were stained with anti-HLA-DR, anti-CD123, and AHlgE monoclonal antibodies. HLA-DR-negative/CD123-positive cells in the forward scatter versus side scatter blast cell window were identified as basophils, and human IgE expression was determined. The expression of cell surface IgE on basophils after each step is presented in: (a) healthy volunteer basophils, (b) basophils treated with lactic acid, and (c) basophils subsequently treated with third-party plasma.

anti-CD123 MoAb. After washing with PBS/BSA/EDTA, the cells were treated with the FACS lysis solution and subjected to FCM. HLA-DR-negative and CD123-positive cells in the forward versus side scatter blast cell window were identified as basophils. CD203c was used as a marker of basophil activation.^{14,15}

To calculate the percentage of CD203c-positive basophils in each sample, a cursor was placed on the CD203c histograms at the point at which 5% of the basophils were positive for CD203c without stimulation. When the percentage of CD203c-positive basophils after stimulation by each PC-SN exceeded 20%, the specific PC-SN was categorized as BAT-positive.¹⁵

Statistical analysis

The Wilcoxon signed-ranks test with Bonferroni correction was used to determine statistical significance. A p value <0.05 was considered statistically significant.

RESULTS

Evaluation of the pi-BAT using quasi-basophils

The pi-BAT consists of two processes: pretreatment and functional assessment (Fig. 1).

Pretreatment (dissociation and replacement of IgE)

To confirm that IgE of basophils' own origin was replaced with that of third-party origin, we measured surface IgE expression during each step using FCM. As shown in Fig. 2, treatment with lactic acid dissociated almost all surface IgE (Fig. 2a,b); and treatment with third-party plasma reconstituted the cell-bound IgE completely (Fig. 2c).

Functional assessment

To examine whether pretreatment hampered the allergen/IgE-dependent activation of quasi-basophils, AHlgE MoAb, which is known as a strong basophil stimulator through the IgE-dependent pathway, was used to stimulate the basophils, and the extent of CD203c expression on the quasi-basophils was analyzed using FCM. MoAb concentrations between 5 and 0.01 $\mu\text{g/mL}$ had no effect on the activation of either untreated basophils or quasi-basophils, as measured by the elevated expression of CD203c (IgE from healthy laboratory personnel). However, 0.5 $\mu\text{g/mL}$ AHlgE induced a slightly lower activation of quasi-basophils than did 5 $\mu\text{g/mL}$ AHlgE MoAb, which was significant ($p = 0.02$); this difference was not observed in untreated basophils (Fig. 3a,b).

Because an allergen/IgE-dependent allergic reaction is induced by the cross-linking of antigen-specific IgE bound to high-affinity IgE receptors on basophils (mast cells), we investigated whether quasi-basophils were activated by relevant (but not irrelevant) antigen. Surface IgE was replaced by third-party plasma that contained no specific IgE against house dust or Japanese cedar pollen (Type A), house dust-specific IgE (Type B), or Japanese cedar pollen plus house dust-specific IgE (Type C). Quasi-basophils were then stimulated with relevant or irrelevant antigens. The first group of quasi-basophils (IgE, Type A plasma) was not activated by either Japanese cedar pollen or house dust (Fig. 4a,b). The next group of quasi-basophils (IgE, type B plasma) was activated by house dust but not by Japanese cedar pollen (Fig. 4c,d), whereas the third group of quasi-basophils (IgE, type C plasma) was activated by both antigens (Fig. 4e,f). Thus, the activation was

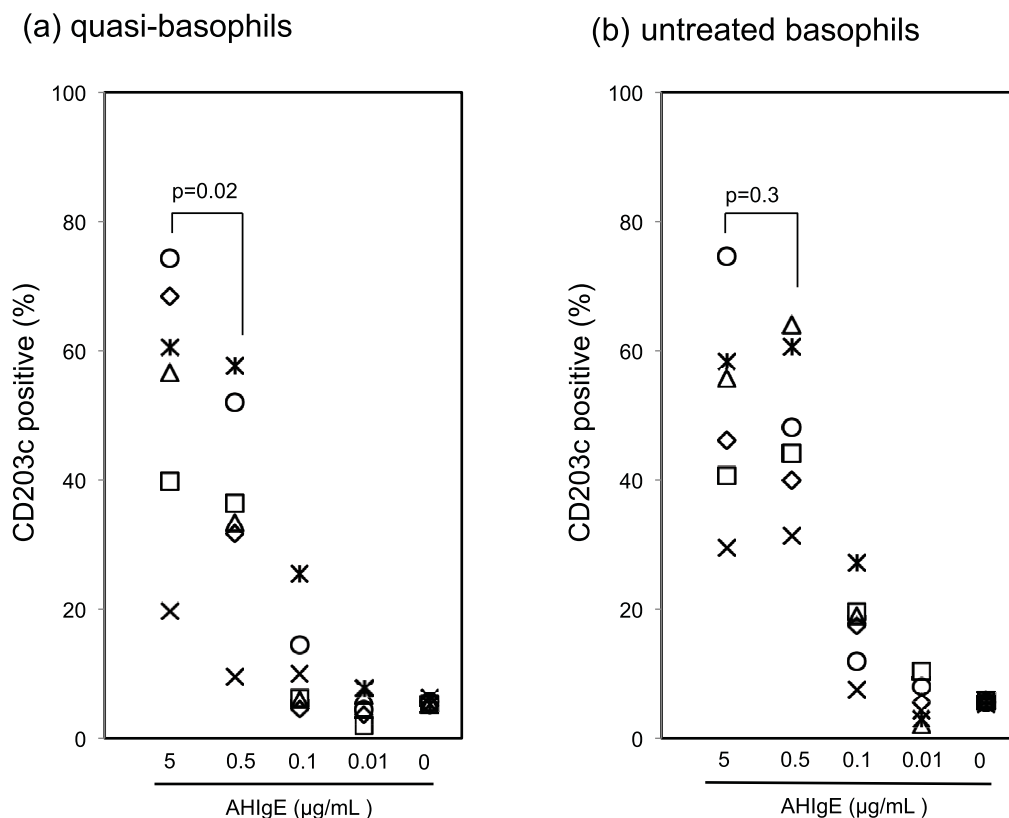


Fig. 3. Comparison of activation between quasi-basophils and untreated basophils. Peripheral blood mononuclear cells were collected from six individual healthy volunteers. Each sample ($N = 6$) was divided into two equal volumes: One fraction was used as the source of untreated basophils, and the other was treated with lactic acid and third-party plasma (quasi-basophils). Both (a) quasi-basophils and (b) untreated basophils were stimulated with several concentrations of AHlgE.

elicited only by relevant antigens. We further confirmed these results in two other responder cells (data not shown). The results from these experiments indicate that quasi-basophils can be activated like untreated basophils when stimulated by relevant antigens.

Having confirmed the activation of quasi-basophils by an allergen/IgE-dependent pathway in an antigen-specific manner, we next examined their activation by an allergen/IgE-independent pathway using soluble bioactive molecules, including LPS, rhIL-3, fMLP, and rhIL-33.^{7,14,15} We compared the activation of quasi-basophils (IgE, plasma from the same source as basophils) with that of untreated basophils in response to these molecules. As shown in Fig. 5, the quasi-basophils exhibited much less activation than untreated basophils in response to rhIL-3 and rhIL-33 (Fig. 5a,b) but equivalent activation in response to fMLP. However, both untreated basophils and quasi-basophils exhibited enhanced activation in response to LPS. Thus, the activation of quasi-basophils by an allergen/IgE-independent pathway was inconsistent and depended on the particular agonist added.

Analysis of two ATR cases using the pi-BAT

We first performed the BAT for two patients with PC transfusion-related ATR. The results of the BAT performed on Case 1 were previously reported.¹⁶ In Case 2, which was first reported in this study, the BAT was delayed 40 days after a transfusion-related anaphylaxis episode due to myelosuppression. Basophil activation in response to PC-SNs was elicited when the patient's blood sample (Fig. 6a), but not a healthy volunteer's blood sample, was used as the source of basophils (Fig. 6b,c). Similar results also were obtained 151 days after the anaphylaxis episode. This basophil activation was inhibited by dasatinib, indicating that basophil activation occurred via an IgE-dependent pathway (Fig. 6).^{15,22} Having demonstrated that the BAT was positive in both cases and that basophil activation was elicited by an allergen/IgE-dependent pathway, we performed the pi-BAT for these patients. Because this was a retrospective study, patient sera were not obtained during the ATR episodes but were collected when the BAT was performed. As shown in Figure 7, upon stimulation with PC-SNs, the pi-BAT was positive when the patient's IgE (Fig. 7b,d), but not a third-party IgE, was used to replace

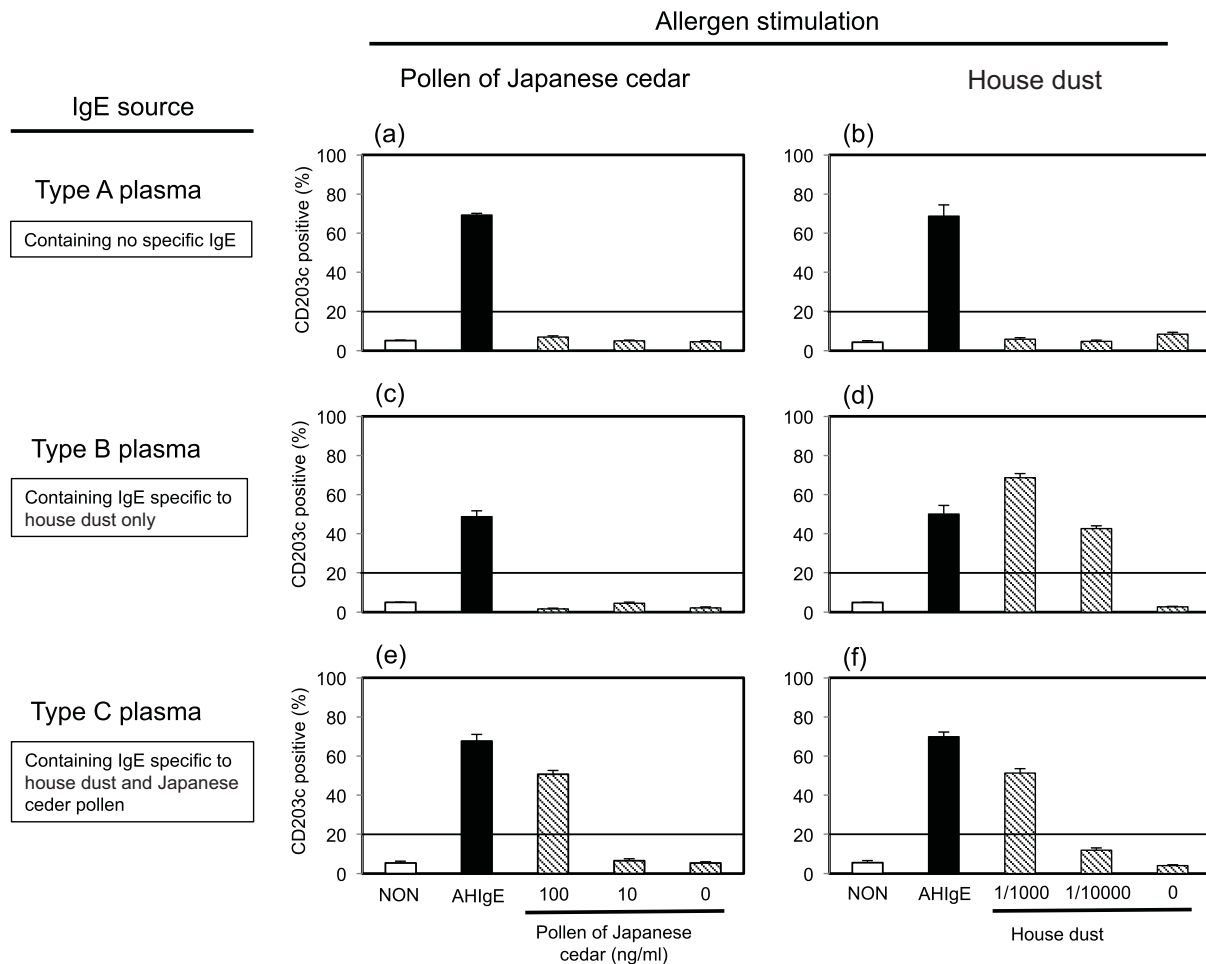


Fig. 4. Reconstruction of allergen specificity in quasi-basophils. Lactic acid-treated basophils were resuspended in three types of third-party plasma: Quasi-basophil IgE was stimulated with Japanese cedar pollen at two concentrations (100 ng/mL or 10 ng/mL), house dust at two dilution ratios (1:1000 or 1:10,000), anti-human IgE as a positive control, and phosphate-buffered saline as a negative control. The activation of quasi-basophils was correlated to expression levels of CD203c. When the percentage of CD203c-positive basophils exceeded 20% (solid line), PC-SNs were considered positive. The expression ratios of CD203c for each quasi-basophil group are shown in (a) through (f). All results are presented as means $\pm$ standard deviations (N = 3). NON = no agonist.

the IgE on the responder basophils in both cases (Fig. 7a,c).

DISCUSSION

We previously reported that the BAT was used as a functional test in transfusion medicine to assess the causal relationship between ATRs and the transfusion and to examine whether the reactions occurred through an allergen/IgE-dependent or allergen/IgE-independent pathway.¹⁴⁻¹⁶ Moreover, both pathways of basophil activation can be assessed by the BAT.^{15,16,23} However, those studies had several limitations. One was that the patients who required a transfusion, especially PCs, experienced myelosuppression, so that their numbers of basophils were insufficient to perform the BAT. To address this issue in

our study, we applied the pi-BAT, in which patient serum/plasma is used as the source of patient IgE, residually transfused blood is used as the allergen source, and healthy volunteer basophils are used as the source of responder cells.¹⁷⁻¹⁹ In both of our patients, PC-SNs prepared from residually transfused PCs exclusively activated quasi-basophils that expressed patient IgE, and not IgE from healthy volunteers. These results indicate that the pi-BAT can be used to clarify the causal relationship between ATRs and transfused blood.

To apply the pi-BAT in the clinic, one must prepare quasi-basophils, which involves the dissociation of their receptor-bound IgE. Pruzansky and coworkers first reported a simple dissociation method: basophils were stripped of their own surface IgE by suspending them in a simple lactic acid buffer at pH 3.7 for 3 minutes at room

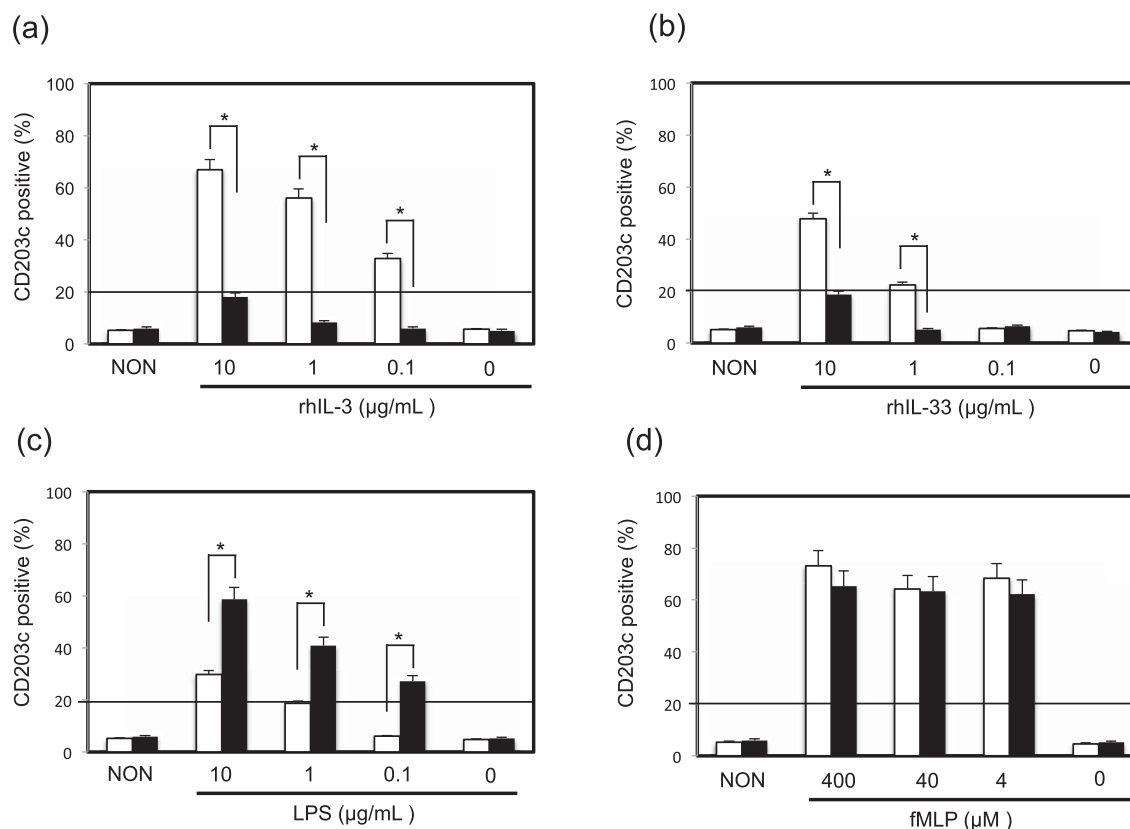


Fig. 5. Differences in the activation of untreated basophils and quasi-basophils through an allergen/IgE-independent pathway. Untreated basophils (white bars) and quasi-basophils (black bars) were stimulated with (a) rhIL-3 (10, 1, or 0.1 $\mu\text{g/mL}$), (b) rhIL-33 (10, 1, or 0.1 $\mu\text{g/mL}$), (c) LPS (10, 1, or 0.1 $\mu\text{g/mL}$), (d) fMLP (400, 40, or 4 μM), or phosphate-buffered saline. All results are presented as means $\pm$ standard deviations ($N = 3$). Basophil activation was correlated to the expression levels of CD203c. * $p < 0.01$.

temperature.¹⁸ The dissociation ratio for this method was between 50% and 90%, and it had no significant effect on basophil activation. Santos and colleagues reported that lymphocytes, which were isolated from whole blood using density-gradient centrifugation with histopaque-1077, were depleted of their receptor-bound IgE after treatment with lactic acid at 4°C for 5 minutes.¹⁹ Those authors demonstrated that these quasi-basophils, in which the IgE was replaced after treatment with patient plasma, reproduced the patient's specific allergy. Based on these two studies, we improved the dissociation step by using HES40 as a PBMC separator and lactic acid as an IgE dissociation agent; this modification achieved a higher dissociation rate of endogenous IgE (>98%) and improved basophil viability (>98%) without any significant effects on the activation of IgE-reconstituted basophils. The differences between these methods are summarized in Table 2.

Although we performed the BAT for two ATR cases, and both cases tested positive 2 weeks (Case 1) and 40 days (Case 2) after the ATR episode, it is not clear when the BAT should be performed for optimal ATR analyses. In Case 1, the BAT was still positive 2.5 months after the ATR

episode but was negative 7 months after the ATR (unpublished data). In Case 2, the BAT was still positive 5 months after the ATR episode. In general, allergy persists throughout life; however, IgE-positive B cells rarely differentiate into memory B cells or long-lived plasma cells, and basophils have a short life span, averaging 2 to 5 days.^{24,25} Furthermore, the half-life of IgE in serum is less than a few days, suggesting that allergy may not last very long.²⁴ Considering these conflicting findings, it may be desirable to perform the BAT soon after an ATR episode, because qualitative changes in patient basophils and serum IgE may occur. The best time to perform the BAT must be determined in future studies. In contrast, the pi-BAT can be performed whenever patient serum is available, commonly just before or around the ATR episode.

It was difficult to determine the temperature under which (4°C or room temperature [RT]) the residual PCs should be preserved and centrifuged to prepare PS-SN samples. We were in a dilemma, because, at 4°C, there was a possibility that the residual platelets would release some biologic response modifiers (BRMs), which are known to be associated with platelet cold activation; whereas at RT, there was a similar possibility that the

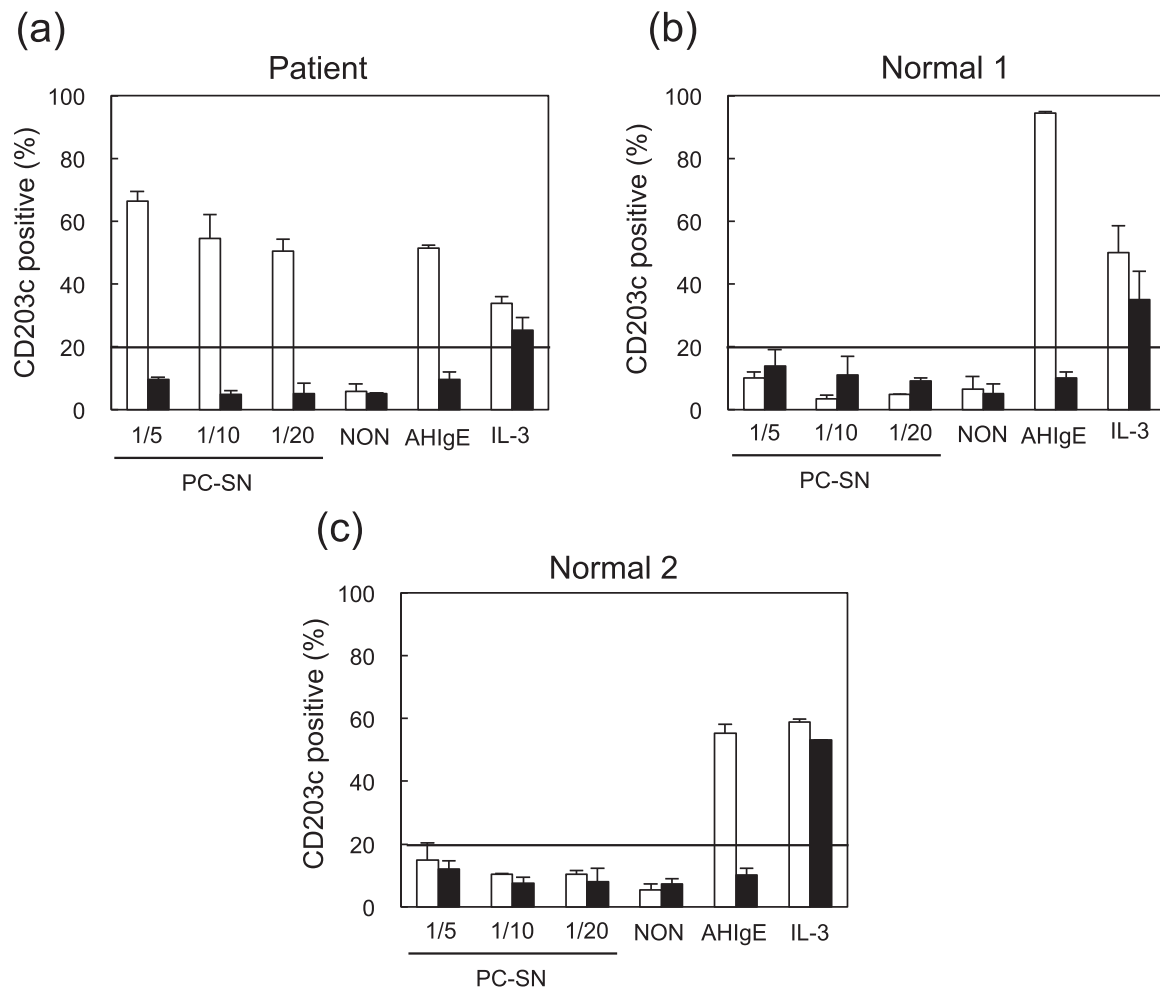


Fig. 6. Basophil activation by supernatants of the residual PC-SNs for Case 2. Anti-human IgE monoclonal antibody was used as a positive control that could be inhibited by dasatinib; IL-3 was used as another positive control that could not be inhibited by dasatinib. (a) Whole blood from Case 2 (patient) and (b,c) from two healthy volunteers (Normal 1 and Normal 2, respectively) was preincubated in the absence (white bars) or presence (black bars) of dasatinib and subsequently incubated with patient PC-SNs at several dilution ratios (1/5, 1/10, and 1/20). When the percentage of CD203c-positive basophils exceeded 20% (solid line), the PC-SNs were considered positive. This patient's PC-SNs exclusively activated their own basophils, and the activation was inhibited by dasatinib.

platelets would keep releasing some other preservation-associated BRMs. When we started this study, we selected 4°C for the following four reasons: First, Reddoch and coworkers reported that cold-stored platelets released lower concentrations of BRMs than RT-stored platelets, although the various markers of platelet activation, including CD62P expression, phosphatidylserine exposure, and CD40L expression, increased during cold storage compared with RT storage.²⁶ Second, we previously demonstrated that the level of mitochondrial damage-associated molecular patterns, which are one of the BRMs released from activated platelets, showed no significant quantitative difference between preservation and centrifugation at 4°C and at RT (unpublished data). Third, we confirmed that there was no significant difference between

the two conditions in the activation ability of the PC-SNs onto neutrophils, monocytes, or basophils (unpublished data). Fourth, the cold preservation of the residual PCs may restrain bacterial proliferation if they are contaminated with bacteria, although this possibility is quite low.

However, the pi-BAT can still be improved. First, patient sera used for the pi-BAT in this study were not obtained before or around the ATR episodes but were collected from the remaining sera after the BAT was performed. For future studies, we must confirm that the pi-BAT actually works optimally with sera obtained before or around the time of ATR episodes. Second, the sensitivity and specificity of the pi-BAT remain undetermined, because we have only performed it in two patients with ATR. Furthermore, there are no data about the relationship

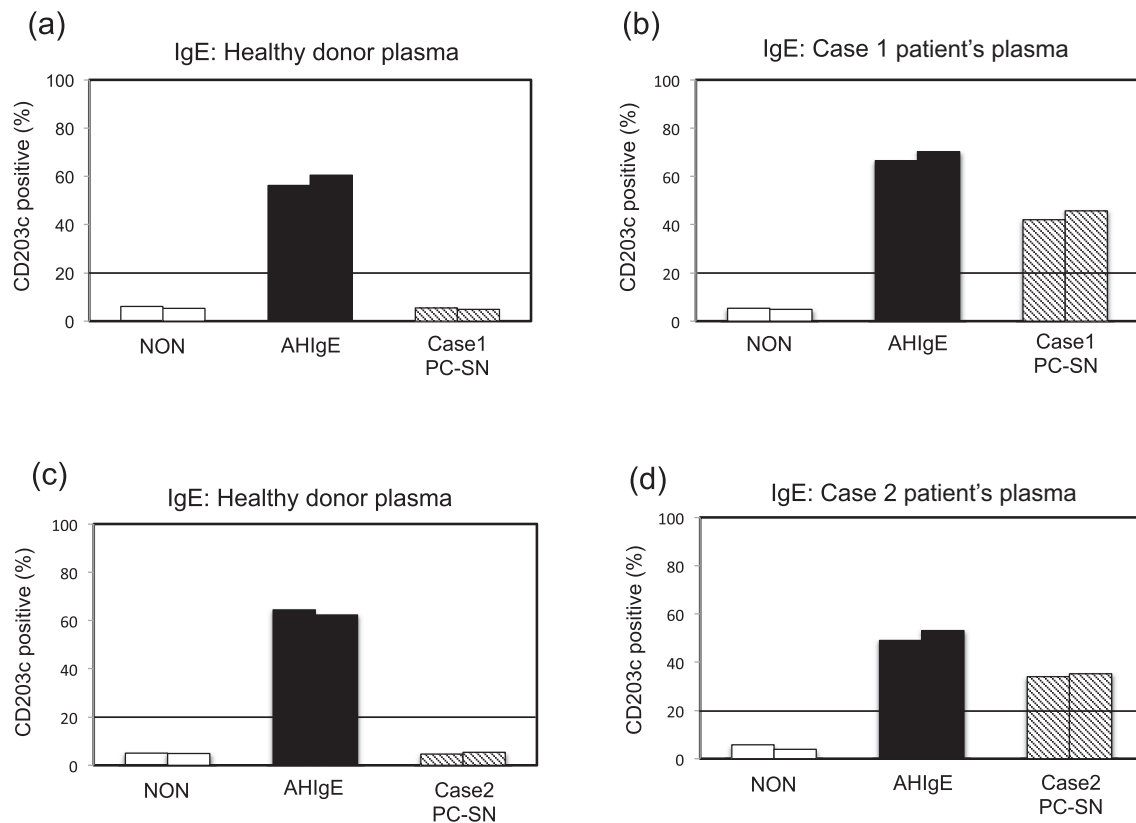


Fig. 7. Activation of quasi-basophils by supernatants of the residual PC-SNs from Cases 1 and 2. Lactic acid-treated basophils were resuspended in plasma from Case 1, Case 2, or a healthy donor. The quasi-basophils were stimulated with the specific patient's PC-SNs or with anti-human IgE. Quasi-basophils were treated (a) with healthy donor plasma then stimulated with Case 1 PC-SNs, or (b) with Case 1 plasma then stimulated with Case 1 PC-SNs, or (c) with healthy donor plasma then stimulated with Case 2 PC-SN, or (d) with Case 2 plasma then stimulated with Case 2 PC-SNs. Duplicates were performed for all combinations described.

TABLE 2. Modification of dissociation step

Method	Isolated cell fraction	Dissociation reagent	Dissociation ratio of own IgE	Basophil viability	Activation ability of IgE-reconstituted basophils
Pruzansky's method	Lymphocyte fraction	Lactic acid buffer	50-90%	NT	+
Santos' method	Lymphocyte fraction	Lactic acid	NT	NT	+
Presented method	Mononuclear cell fraction	Lactic acid	>98%	>98%	+

NT = not tested.

between ATR severity and the level of basophil activation. Therefore, similar but larger studies are required to conclusively evaluate the pi-BAT. Third, because previous reports studied patient basophils that were previously primed by stimulation factors, including cytokines, chemokines, and damage-associated molecular patterns, it is possible that the activation of healthy volunteer basophils, which we used as a source of quasi-basophils, may not be as high as that of patient basophils.^{23,27,28} Furthermore, the process of preparing quasi-basophils slightly affects basophil activation (Fig. 3). These results indicate that the pi-BAT may not

be as sensitive as the BAT. Third, the pi-BAT is not suitable for the analysis of an allergen/IgE-independent pathway, because the pretreatment hampers reactivity to some BRMs (i.e., IL-3 and IL-33) (Fig. 5). For this kind of analysis, the BAT is suitable. Fourth, we wished to examine whether PCs that did not cause a reaction would test negative with the pi-BAT; however, unfortunately, we could not do this for the following two reasons; 1) it was very difficult to obtain PCs without a reaction, and 2) the patients' plasma samples from both Case 1 and Case 2 were of insufficient quantity to perform additional experiments. Fifth, there

are several biomarkers to detect basophil activation. Leukotriene production and calcium influx are representative markers of activated leukocytes, but they are not specific to basophils. Histamine is released from activated basophils and mast cells and is one of the profitable biomarkers of basophil activation. However, the detection sensitivity of histamine may not be high enough, because basophils represent only 1 to 2% of white blood cells, with the result that the released histamine is diluted too much when using whole blood as a basophil source. In this respect, the determination of surface-activation markers of basophils, such as CD63, CD69, and CD203c, would be more sensitive. However, a previous study reported that CD69 was up regulated over the course of 4 to 24 hours by agonist stimulation.²⁹ In most ATR cases, in contrast, the symptoms are usually observed within 4 hours after the transfusion, indicating that CD69 may not be suitable to determine the basophil activation related to ATRs.

Although CD63 corresponds to the early activation of basophils, it is also an activation marker of platelets. Platelet activation leads to the up regulation of not only P-selectin but also CD63 and to the production of platelet microparticles with P-selectin and CD63 on their surface. These activated platelets and microparticles can bind to basophils through their cell surface PSGL-1, a ligand of P-selectin, leading to a false-positive result (unpublished data). In contrast, CD203c is expressed only by basophils and their precursors. In addition, CD63 was less sensitive than CD203c in our previous studies. Therefore, we believe that CD203c is a better basophil-activation marker than CD63 when whole blood is used as a basophil source. However, the up regulation of CD203c correlates with basophil activation but not directly with degranulation. This is the greatest limitation of CD203c as a biomarker for basophil activation. Furthermore, although whole blood used for the pi-BAT was acquired from healthy laboratory personnel employed at our blood center, and the Ethics Committee of the Japanese Red Cross Society approved the study, there still may be ethical considerations. Because of the advantages and disadvantages of the pi-BAT, we recommend using the BAT and the pi-BAT synergistically in the clinic.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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