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ORIGINAL ARTICLE: CLINICAL

Expression of CD203c on basophils as a marker of immunoglobulin E-mediated L-asparaginase allergy

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Abstract

Immediate allergy to L-asparaginase (ASP) is a major obstacle in treating lymphoid malignancies. ASP-specific immunoglobulin G (ASP-IgG) has been used as a surrogate marker. Recently, the CD203c-basophil activation test (BAT) was found to be useful in diagnosing IgE-mediated allergies. We compared the diagnostic utility of the CD203c-BAT to that of ASP-IgG levels in determining ASP allergies in children. Eight ASP allergic reactions occurred over 75 ASP treatment courses. The sensitivity, specificity and area under the receiver operating characteristic curve of CD203c-BAT were similar to the ASP-IgG levels (0.75 vs. 0.85, 0.82 vs. 0.78 and 0.81 vs. 0.85, respectively). Positive skin prick test results in patients with ASP allergy suggested that ASP-IgE was one of the key players in ASP allergy. A combination of the BAT with the ASP-IgG level had the highest specificity (0.95) and positive predictive value (0.62), which permitted us to identify ASP allergy more effectively.

Keywords: L-asparaginase, IgE-mediated allergy, basophil activation test

Introduction

L-asparaginase (ASP) is a key drug for treating pediatric acute lymphoblastic leukemia (ALL) and lymphoblastic lymphoma (LBL) [1–5]. Because ASP is not expressed in normal human cells, approximately 10–45% of patients develop immediate hypersensitivity [6,7]. ASP allergy is sometimes severe [7], and after an allergic reaction occurs, it is recommended that the patient discontinues ASP therapy or converts from the first-line formulation (i.e. *Escherichia coli*-derived native ASP, polyethylene glycolated ASP) to the second-line formulation

(i.e. *Erwinia*-derived ASP). Because *Erwinia*-derived ASP is only available in limited locations and the optimal dosing of *Erwinia*-derived ASP is controversial, *E. coli*-derived ASP has maintained its position as first-line treatment over the past several decades [8]. Thus, it is important to know whether patients have *E. coli*-derived ASP allergy. Although immediate allergy is generally mediated by specific immunoglobulin E (IgE), ASP-specific IgE is known to be difficult to detect; thus, ASP-specific IgG has been used as a surrogate marker to identify ASP allergy in a clinical setting [9–11]. However, in previous reports, ASP-specific IgG has been detected not only in patients with ASP allergy but also in non-allergic patients treated with ASP. Therefore, the utility of measuring ASP-specific IgG levels for diagnosing ASP allergy is still controversial [11]. In contrast, the skin prick test (SPT) is considered to be the standard for diagnosing immediate hypersensitivity. However, the SPT is an *in vivo* test and occasionally provokes severe allergic reactions [12].

To avoid unnecessary discontinuation or conversion of ASP therapy, an accurate *ex vivo* marker of ASP allergy is needed. Recently, several diagnostic tests using IgE-mediated allergy effector cells have been developed [13]. The basophil activation test (BAT), in which the surface expression of the degranulation/activation marker (CD203c) on basophils is detected, is known as a reliable tool for diagnosing IgE-mediated allergies; the BAT is an *ex vivo* test, has a high sensitivity and specificity, can be used to test any allergen and is quantitative [14]. However, using the BAT to diagnose ASP allergy in pediatric patients with malignancy has not been assessed.

In this study, we evaluated the diagnostic utility of the BAT and ASP-specific IgG. Moreover, using the skin prick test, we examined whether ASP allergy was mediated by specific IgE.

Patients and methods

Patients

A total of 32 children diagnosed with ALL or LBL were studied between June 2010 and October 2012 (Table I). The patients were treated according to modified protocol L99-15 of the Tokyo Children's Cancer Study Group (Supplementary Table I to be found online at <http://informahealthcare.com/doi/abs/10.3109/10428194.2013.794944>). The patients with ALL were placed into high-risk (HR), intermediate-risk (IR) or low-risk (LR) groups based on their age, leukocyte count at diagnosis, immunophenotype, prednisolone response and cytogenetic profile [15]. The patients with LBL were treated according to a modified IR regimen, and patients with relapsed ALL were treated according to the ALL-BFM95 regimen [16]. We collected heparinized whole blood samples and sera before the ASP-containing treatment or within 30 days after recovery from bone marrow suppression, and grouped test results according to the reaction to ASP treatment: "ASP allergy" samples or "non-allergy" samples. This study was approved by the institutional review board, and written informed consent was obtained from the patients' guardians.

Diagnostic criteria for ASP allergy

Patients were diagnosed with ASP allergy and added to the "ASP allergy" group when an allergic reaction occurred within several hours after an ASP injection. Patients who tolerated the ASP injection were placed in the "non-allergy" group. The severity of the allergy was classified as mild (only urticaria or rash), moderate (bronchospasm, gastrointestinal symptoms or allergy-related edema/angioedema with or without urticaria) or severe (anaphylactic shock) (Table I).

Skin prick test

L-asparaginase (Leunase®) was kindly provided by Kyowa Kirin (Tokyo, Japan). Because our treatment protocol used ASP at a concentration of 30 µM by intramuscular injection,

SPTs were performed using ASP at the same concentration (30 µM). A positive control (1% histamine) and a negative control (saline) were also included. Reactions were assessed at 15 min based on the size of the wheal. A wheal with a diameter more than 3 mm greater than the diameter of the negative control was considered to be positive. Because three of eight ASP-allergic patients and one of the non-allergic patients refused the SPT, SPTs were examined in five ASP allergic reactions and 66 non-allergic reactions. To date, no systemic allergic reactions, such as anaphylaxis, have been reported following an ASP-SPT.

Basophil activation test

A commercial kit (AllergenCity Kit; Beckman Coulter, Miami, FL) was used to quantify CD203c expression in basophils according to the manufacturer's instructions. Briefly, heparinized whole blood samples were incubated with different ASP concentrations (0.06–15 µM) for 30 min. An anti-IgE antibody (positive control) and phosphate buffered saline (PBS; negative control) were also incubated with heparinized whole blood samples. Staining reactions were performed at 37°C. Because chemoattractant-homologous receptor expressed on T helper type 2 (Th2) cells (CRTH2, a prostaglandin D2 receptor) is expressed not only on Th2 lymphocytes but also on basophils and eosinophils, the basophils were quantified as SSC^{low}, CD3[–] and CRTH2⁺ using a side-scatter plot, anti-CD3-paired basic amino acid cleaving enzyme-7 (PC7) (UCHT1) and anti-CRTH2-fluorescein isothiocyanate (FITC) (BM16), respectively. CD203c expression on basophils was examined using anti-CD203c-phycoerythrin (PE) (97A6) (Supplementary Figure 1 to be found online at <http://informahealthcare.com/doi/abs/10.3109/10428194.2013.794944>). The data were acquired using a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA) equipped with lasers at 488 nm, 633 nm and 405 nm. Spectral overlap was compensated using FACSDiva software (BD Biosciences) and analyzed using FlowJo software (TreeStar Inc., Ashland, OR). CD203c up-regulation on basophils was determined using a threshold that was defined by the fluorescence of unstimulated cells (negative control, Supplementary Figure 1 to be found online at <http://informahealthcare.com/doi/abs/10.3109/10428194.2013.794944>).

Measurement of ASP-specific IgG levels using ELISA

Sera obtained on the BAT analysis day were frozen at –30°C and then used to measure the ASP-specific IgG levels. We modified the assay published by Woo *et al.* [10] to determine the level of ASP-specific IgG. Briefly, microplates (Nunc 442404, F96 MaxiSorp) were coated with 0.35 µM ASP diluted in a carbonate/bicarbonate buffer at pH 9.5 and incubated for 2 h at room temperature. The plates were washed with PBS containing 0.1% Tween-20 and then blocked with PBS containing 0.5% bovine serum albumin (BSA), 5% fetal bovine serum (FBS) and 0.1% Tween-20. Serially diluted serum samples were added and incubated for 2 h at room temperature. Biotin-conjugated monoclonal anti-human IgG (Becton, Dickinson and Company; BD555785) diluted 1:1000 in PBS containing 0.5% BSA was then added to the samples and incubated for 1 h at room temperature.

Table I. Patient characteristics*.

	Total	ASP allergy	p-Value
No. of patients	32	8	
Median age	10	7.5	0.27
(Range)	(0.4–18.5)	(3.6–17.2)	
Gender			0.20
Female	12	1	
Male	20	7	
Treatment protocol			
ALL-LR	7	1	0.64
IR	14	3	1.00
HR	6	3	0.14
LBL	2	0	1.00
Other	3	1	1.00
Total ASP courses	75	8	
ASP allergy			
Severe	—	3	
Moderate	—	1	
Mild	—	4	

ASP, L-asparaginase; ALL, acute lymphoblastic leukemia; LR, low risk; IR, intermediate risk; HR, high risk; LBL, lymphoblastic lymphoma.

*A total of 32 children diagnosed with ALL or LBL were studied and treated using 75 ASP-containing treatment courses from June 2010 to October 2012. "Other protocol" indicates that the ALL-BFM95 [16] or Japan Pediatric Leukemia/lymphoma Study Group ALL-T11 protocol (in progress) was used. There were eight ASP allergy reactions in eight children.

Thereafter, the staining was developed with horseradish peroxidase (R&D; DY998), and the wells were reacted with a substrate solution (R&D; DY999) for 30 min at room temperature in the dark. After adding 2 N sulfuric acid, the optical density was measured at 450 nm absorbance. The highest titer of a serum sample from an anaphylactic patient was set at 1000 arbitrary units (AU/mL) and used as a positive standard control. Four samples were excluded because of insufficient material.

Statistical analysis

Because some of the quantitative variables were not normally distributed, differences in continuous variables were determined using the non-parametric Mann-Whitney *U*-test, and data were always presented as the median (lower and upper quartile). Categorized variables were analyzed using Fisher's exact test. The discriminative usefulness of the BAT and ASP-specific IgG level was evaluated by calculating the area under the curve (AUC) of receiver operating characteristic (ROC) curves; sensitivity versus 1 – specificity was plotted for each possible cut-off level. Spearman's rank correlation coefficient was used to determine whether the BAT and ASP-specific IgG level were correlated in a monotonic fashion. The statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, San Diego, CA).

Results

Eight ASP allergic reactions occurred in eight of 32 children during the course of 75 ASP-containing treatments. The ages, gender balance and treatment protocols used in the ASP allergy group were not different from those of the non-allergy group. Three of the eight allergic reactions were anaphylactic shock (Table I).

The median ASP-specific IgG titer in the non-allergy group was 194.3 AU/mL (range, 0–4000 AU/mL), while in the ASP allergy group, the median titer was 2158 AU/mL (range, 8.59–4000 AU/mL). As previously reported [11,17], the ASP-specific IgG levels were greater in the ASP allergy group than in the non-allergy group [$p < 0.01$, Figure 1(A)]. In the BAT analysis, the CD203c^{high} basophil rate increased in a dose-dependent manner with the ASP concentration in several BAT-positive patients (Supplementary Figure 2(A) to be found online at <http://informahealthcare.com/doi/abs/10.3109/10428194.2013.794944>). The results at a concentration of 15 μ M ASP were used in the subsequent BAT analyses. The median CD203c^{high} basophil rate on the BAT in the ASP allergy group was 34.2% (range, 3.8–81%), and in the non-allergy group the rate was 9.7% (range, 0.7–50.5%). The CD203c^{high} basophil rate in the ASP allergy group was significantly greater compared to the non-allergy group [$p < 0.01$, Figure 1(B)]. The results of ROC curve analysis revealed that the AUC for the BAT was 0.81 and the AUC for ASP-specific IgG was 0.85 [Figure 1(C)]. The cut-off values for the BAT and ASP-specific IgG level were determined using ROC curve analysis. We set the cut-off value for a positive BAT at 14.4% and the cut-off value for a positive ASP-specific IgG level at 90.7 AU/mL.

Table II shows the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of each test. All of the tests were significantly correlated with

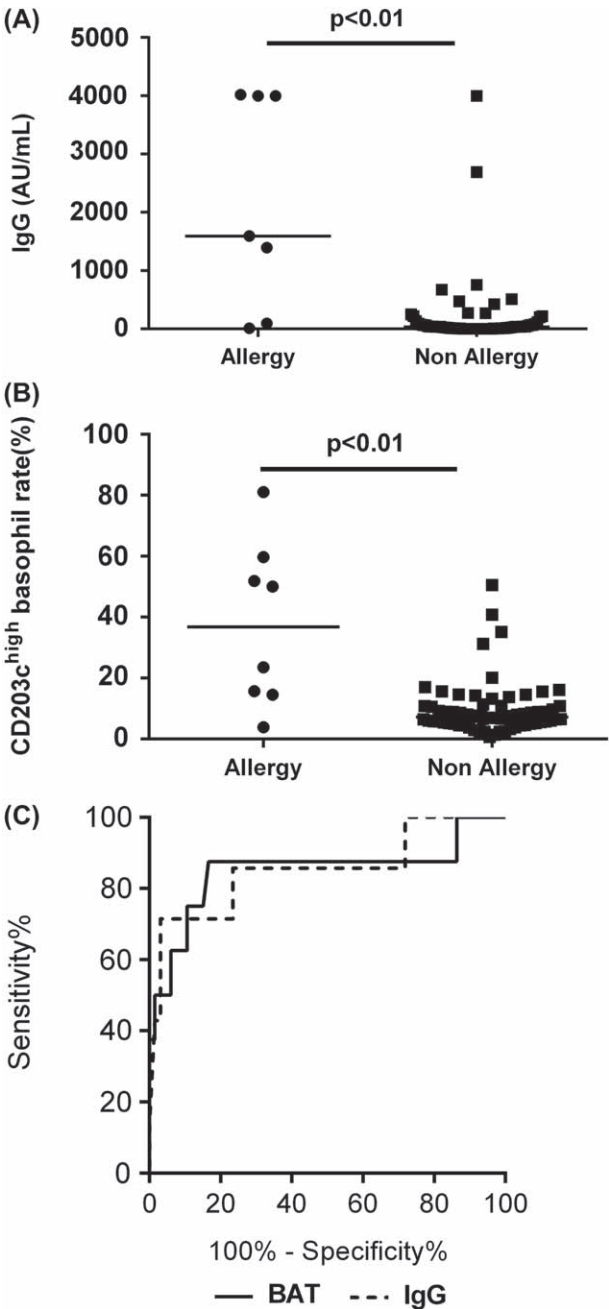


Figure 1. The ASP-specific IgG level (A) and the CD203c^{high} basophil rate in the BAT (B) were significantly greater in the ASP allergy group ($p < 0.01$, Mann-Whitney *U*-test). A ROC curve analysis revealed that the area under the curve (AUC) for the BAT was 0.81 and that the AUC for ASP-specific IgG level was 0.85 (C). We set the positive cut-off value at the score with the shortest distance from the curve to the point (0.0, 1.0). The positive cut-off for the BAT was 14.4%, and the positive cut-off for the ASP-specific IgG level was 90.7 AU/mL.

ASP allergy ($p < 0.01$). The NPV of each test was significantly elevated.

Figure 2 shows the correlation between the CD203c^{high} basophil rate in the BAT (%) and the ASP-specific IgG titer (AU/mL). Spearman's rank correlation coefficient was 0.31 ($p < 0.01$, 95% confidence interval [CI] 0.08–0.51). Our subjects were divided into four groups according to the results of the BAT and ASP-specific IgG assays: (A) BAT (+) ASP-specific IgG (–); (B) BAT (+) ASP-specific IgG (+); (C) BAT (–) ASP-specific IgG (+); and (D) BAT (–) ASP-specific IgG (–).

Table II. Results for the BAT, ASP-specific IgG assay, SPT and combined test results.

	CD203c-BAT		ASP-specific IgG		SPT		BAT + ASP-IgG	
	+	-	+	-	+	-	++	- or +
"ASP allergy" sample (n)	7	1	6	1	3	2	5	2
"Non-allergy" sample (n)	12	55	14	50	1	65	3	61
Fisher's exact test <i>p</i> -value	< 0.01		< 0.01		< 0.01		< 0.01	
Sensitivity (95% CI)	0.75	(0.34–0.96)	0.85	(0.42–0.99)	0.60	(0.15–0.95)	0.71	(0.29–0.96)
Specificity (95% CI)	0.82	(0.70–0.90)	0.78	(0.66–0.87)	0.98	(0.92–0.99)	0.95	(0.86–0.99)
PPV (95% CI)	0.33	(0.13–0.59)	0.30	(0.11–0.54)	0.75	(0.19–0.99)	0.62	(0.24–0.91)
NPV (95% CI)	0.96	(0.87–0.99)	0.98	(0.89–0.99)	0.97	(0.90–0.99)	0.96	(0.89–0.99)

BAT, basophil activation test; ASP, L-asparaginase; IgG, immunoglobulin G; SPT, skin prick test; CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value.

Five of the eight patients with ASP allergic reactions belonged to group B (Figure 2, open circles). The specificity and positive predictive value for group B (BAT+, ASP-specific IgG+) were greater compared to group A or C (Table II).

We followed the CD203c^{high} basophil rate from the BAT in the ASP-allergic patients. Because only *E. coli*-derived ASP was available in Japan, other preparations, such as *Erwinia*-derived ASP or polyethylene glycolated ASP, were not used after the ASP allergic reactions. Interestingly, the CD203c^{high} basophil rate in the BAT decreased just a few months after the observed ASP allergic reactions in four patients (Table III, patients 1, 2, 3 and 7). As expected from the high negative predictive value of the BAT, we were able to safely re-administer ASP to four patients (Table III, patients 1–4) without causing allergy symptoms. We did not re-administer ASP to the other patients with ASP allergies for the following reasons: patient 5 refused ASP re-administration; patient 6 finished the scheduled treatment; patients 7 and 8 underwent stem cell transplants after the ASP allergy reaction. Patients who underwent re-administration were pre-medicated with steroids and antihistamines before the challenge, to avoid severe allergic symptoms. After the first re-administration of ASP, no patient reacted to any of the subsequent doses.

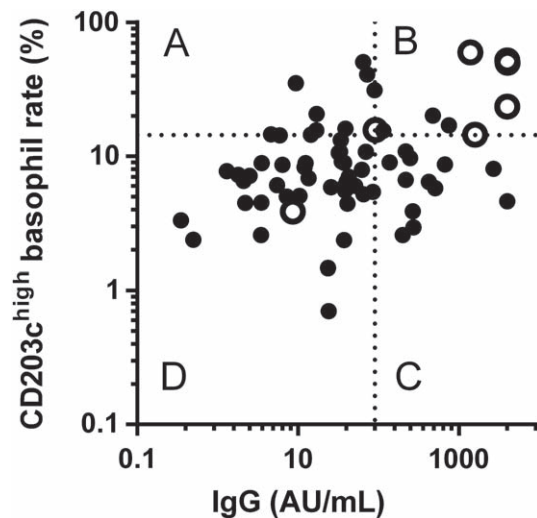


Figure 2. Correlation between the CD203c^{high} basophil rate in the BAT (%) and the ASP-specific IgG level (AU/mL) is shown. Spearman's rank correlation coefficient was 0.31 ($p < 0.01$, 95% CI 0.08–0.51). The dots were divided into four groups: (A) BAT (+) ASP-specific IgG (–), (B) BAT (+) ASP-specific IgG (+), (C) BAT (–) ASP-specific IgG (+), and (D) BAT (–) ASP-specific IgG (–). Five of the eight patients with ASP allergic reactions belonged to group B (open circles). Dashed line represents the positive cut-off value (BAT, 14.4%; IgG, 90.7 AU/mL) for each test.

Discussion

Our results support the hypothesis that the BAT is a useful marker for identifying ASP allergy because its sensitivity and specificity were high, and the AUC of the BAT was as high as the AUC of the ASP-specific IgG level. The dose-dependent effects observed between the ASP concentration and the CD203c^{high} basophil rate in the BAT indicated that basophils were stimulated by ASP. The BAT has been used to assess the functional characteristics of specific IgE in patients. In fact, the low correlation between the BAT and the ASP-specific IgG level indicated by Spearman's rank correlation coefficient ($R = 0.31$, $p < 0.01$) indicated that the CD203c^{high} basophil rate increased independently of the ASP-specific IgG level. The SPT, the standard test to detect IgE, was positive in three of the five patients with ASP allergy with positive BAT, suggesting that the mast cells of allergic patients were activated by ASP-specific IgE. Note that identifying ASP-specific IgE is difficult [18]. Recently, Nakamura *et al.* reported that an IgE crosslinking-induced luciferase expression assay was more sensitive than enzyme-linked immunosorbent assay (ELISA) in identifying specific IgEs [19]. This assay allowed us to detect ASP-specific IgE in a patient with ASP allergy (data not shown). These data indicated that basophils and mast cells were activated by ASP in ASP-allergic patients, and that ASP-specific IgE was one of the key players in mediating immediate ASP allergy. Identifying the CD203c^{high} basophil rate is a promising approach for monitoring ASP allergy.

The BAT is superior to the SPT, particularly in the context of pediatric malignancy, because the SPT is non-quantitative, difficult to compare across different places or times, and

Table III. CD203c^{high} basophil rate in the BAT at time of ASP allergic reaction and after reaction*.

Patient no.	Allergy onset		After allergy		Interval (months)
	Reaction	CD203c-BAT (%)	Reaction	CD203c-BAT (%)	
1	Severe	81	No	8.0	1
2	Moderate	50	No	10.8	3
3	Mild	23.5	No	1.9	1.5
4	Mild	3.85	No	7.6	1
5	Severe	15.7	ND [†]	13.9	5
6	Mild	14.5	ND [‡]	ND	–
7	Mild	23.5	ND [§]	1.97	2
8	Mild	51.9	ND [§]	ND	–

BAT, basophil activation test; ASP, L-asparaginase; ND, not determined.

*CD203c^{high} basophil rate in BAT results for four ASP-allergic patients (nos. 1, 2, 3 and 7) decreased a few months after ASP allergic reaction. We safely re-administered ASP to four patients (nos. 1–4) without allergic symptoms.

[†]Patient 5 refused ASP re-administration.

[‡]Patient 6 finished the scheduled treatment.

[§]Patients 7 and 8 underwent stem cell transplants.

can be affected by any skin condition that the patient might have or medications the patient might be taking. Although our study included no case of anaphylaxis and no patients reported systemic reactions following the ASP-SPTs, a lower burden would be expected because some drug-SPTs induce anaphylaxis. The BAT is performed *ex vivo* and is therefore safe, the results are easy to compare across assays, and it is more quantitative than the SPT.

We had to consider the possibility that basophil activation was mediated not only by specific IgE but also directly by drugs or other pathways. For this reason, we examined the SPTs to confirm the involvement of ASP-specific IgE in ASP allergy. Because ASP allergic reactions without basophil activation were investigated, there were a few non-IgE mediated ASP allergies. ASP allergy was identified most effectively by combining the results of the IgE-mediated BAT and the ASP-specific IgG measurement assays. To date, the low PPV of the ASP-specific IgG level could lead to the unnecessary discontinuation or conversion of ASP therapy. Combining the result of the BAT with the result of the ASP-specific IgG assay would allow clinicians to prepare for the possibility of anaphylaxis. In addition, it is likely that monitoring the ASP-specific IgG level would be useful in assessing ASP neutralization, which is one cause of ASP treatment failure [11].

The BAT is a better marker than ASP-specific IgG level for the follow-up of patients with ASP allergy with positive results, because the half-life of IgE is shorter than that of IgG [20,21]. In fact, because of the rapid, easy operation and high negative predictive value of the BAT, we were able to administer ASP to four patients with ASP allergy without inducing reactions after their BAT results decreased.

The limitations of this study included the small sample size and the lack of ASP activity examinations. Accordingly, a long-term prospective trial should be performed to confirm our findings.

In summary, the BAT is a useful tool for identifying ASP allergy. Combining the BAT with an ASP-specific IgG assay was the most accurate method of identifying ASP allergy. The precise prediction of ASP allergy would help us to avoid severe adverse events and cure more children with lymphoid malignancies.

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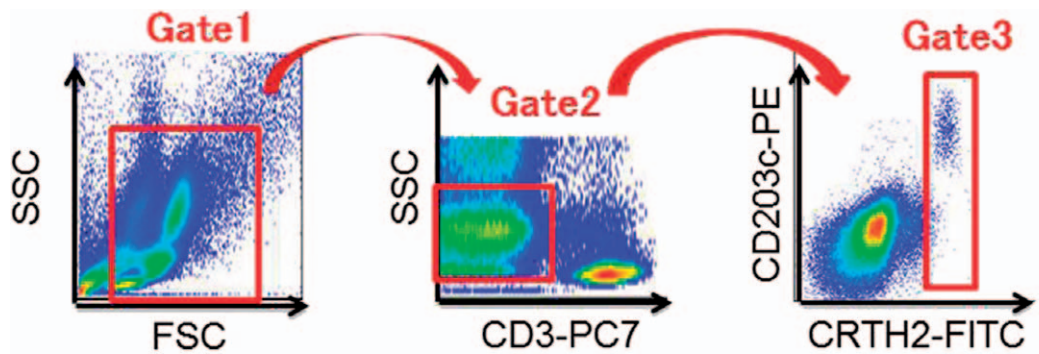
Supplementary material available online

Figures showing further results and table illustrating treatment protocol

Potential conflict of interest: Disclosure forms provided by the authors are available with the full text of this article at www.informahealthcare.com/lal.

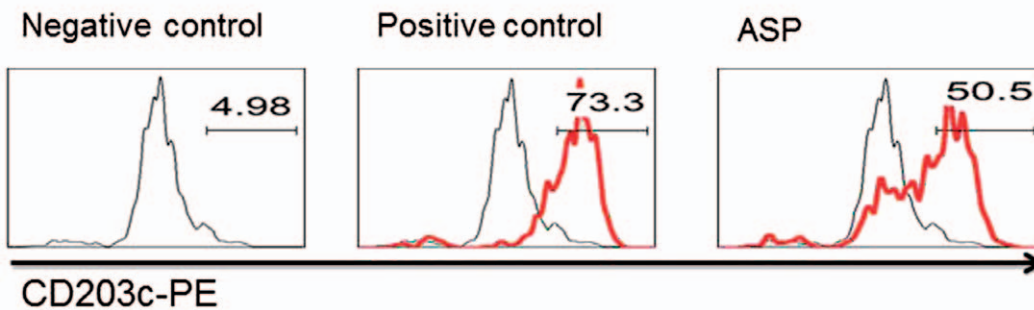
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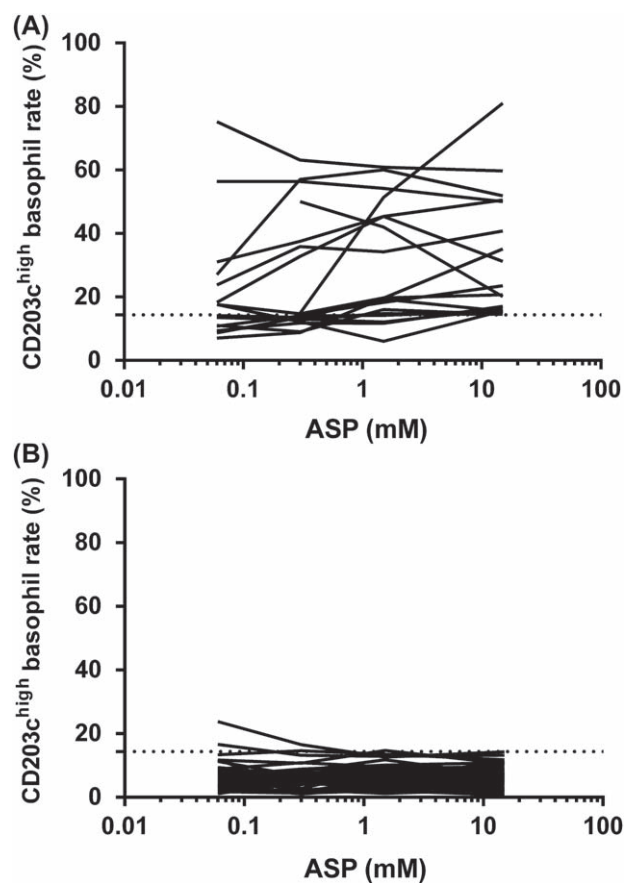
Flowcytometric analysis of allergen-induced expression of CD203c. Basophils were detected on the basis of forward side scatter characteristics (Gate1) and expression of CD3 (Gate2) and positive CRTH2 (Gate3).

The histograms represents basophil gate (Gate3).



CD203c^{high} basophil rate is gated in the top 5% of negative control. CD203c^{high} basophil rate increases after positive control stimulation (anti-IgE). ASP induced enhancement of CD203c^{high} basophil rate in a patient with ASP allergy is observed.

Supplementary Figure 1. Example analysis of the BAT.



Supplementary Figure 2. The CD203c^{high} basophil rate in the BAT increased in a dose-dependent manner with the ASP concentration in several BAT-positive samples (A). The CD203c^{high} basophil rates in the BAT results for the 56 BAT-negative samples are shown (B). The dashed line represents the positive cut-off value (14.4%).

Supplementary Table I.

modified TCCSG L99-15 protocol**Low risk (LR)**

Induction A: PSL 60 mg/m²×5 weeks, VCR 1.5 mg/m²×5, THP-ADR 20 mg/m²×2, ASP 6,000 U/m² IV×9

Intensification A: CY 1,000 mg/m², Ara-C 75 mg/m², SC×15, 6MP 60 mg/m²×21

CNS prophylaxis: MTX 3 g/m²×3

Interim maintenance: 6MP 60 mg/m²×14, MTX 25 mg/m²×3

Reinduction: PSL 60 mg/m²×14, VCR 1.5 mg/m²×3, THP-ADR 20 mg/m²×3, ASP 10,000 U/m² SC×4

Late intensification 1: 6MP 60 mg/m²×14, CY 1,000 mg/m², Ara-C 75 mg/m²×10

Late intensification 2 (3cycle): MTX 500 mg/m², PSL 40 mg/m²×14, VCR 1.5 mg/m²×3, ASP 10,000 U/m² SC

Maintenance: 6MP/MTX 2 years

Total number of IT therapies: 11

Intermediate risk (IR)

Induction B: PSL 60 mg/m²×5 weeks, VCR 1.5 mg/m²×5, Daunomycin 25 mg/m²×4, CY 1,000 mg/m²×2, ASP 6,000 U/m² IV×9

Intensification A : equal to standard risk

CNS prophylaxis: equal to standard risk

Reinduction: DEXA 6 mg/m²×14, VCR 1.5 mg/m²×3, Adriamycin 25 mg/m²×4, ASP 10,000 U/m² SC×4

Late intensification (2 cycle):

Ara-C 75 g/m²×10, 6MP 60 mg/m²×14, CY 10,000 mg/m²

Ara-C 2 g/m²×4, ASP 10,000 U/m² SC

PSL 40 mg/m²×14, VCR 1.5 mg/m²×3, THP-ADR 20 mg/m²×3, ASP 10,000 U/m² SC×2

MTX 500 mg/m²

Maintenance: 6MP/MTX 1 years

Total number of IT therapies: 11

High risk (HR)

Induction B: equal to high risk

Intensification A : equal to standard risk

Intensification BFM HR 1-1: DEXA 20 mg/m²×5, MTX 5 g/m², CY 200 mg/m²×5, Ara-C 2 g/m²×2, ASP 25,000 U/m² IM

Intensification BFM HR 2-1: DEXA 20 mg/m²×5, Vindesine 3 mg/m²×2, Daunomycin 30 mg/m², MTX 5 g/m², IFO 800 mg/m²×5, ASP 25,000 U/m² IM

Intensification BFM HR 3-1: DEXA 20 mg/m²×5, Ara-C 2,000 mg/m²×4, Vp-16 100 mg/m²×5, ASP 25,000 U/m² IM

Intensification BFM HR 1-2:

Intensification BFM HR 2-2:

Intensification BFM HR 3-2:

Late intensification (BFM protocol II)

DEXA 10 mg/m²×14, VCR 1.5 mg/m²×4, Adriamycin 30 mg/m²×4, ASP 10,000 U/m² IM×4

6MP 60 mg/m², CY 1 g/m², Ara-C 75 mg/m²×8

Maintenance: 6MP/MTX 1 years

Total number of IT therapies: 10

The prophylactic irradiation dose was 12Gy and therapeutic irradiation dose for patients with initial involvement of the central nervous system was 12 Gy for patients 1 to 2 years and 18 Gy for patients more than 2 years of age.
PSL, prednisone; VCR, vincristine; ASP, *E coli* L-asparaginase (Leunase™, KYOWAKIRIN, Tokyo); IV, intravenous; SC, subcutaneous; IM, intramuscular; MTX, methotrexate; 6-MP, 6-mercaptopurine; Ara-C, cytarabine; CY, cyclophosphamide; DEXA, dexamethasone; IFO, ifosfamide; IT, intrathecal therapy

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