

Original Article

Basophil Activation Test in the Diagnosis and Monitoring of Mastocytosis Patients with Wasp Venom Allergy on Immunotherapy

Katayoon Bidad,^{1,2} Martijn C. Nawijn,^{2,3} Antoon J. M. van Oosterhout,^{2,3} Sicco van der Heide,^{3,4} and Joanne N. G. Oude Elberink^{3,5*}

¹Immunology, Asthma and Allergy Research Institute, Tehran University of Medical Sciences, Tehran, Iran

²Department of Pathology and Medical Biology, Laboratory of Allergology and Pulmonary Diseases, University Medical Center of Groningen, University of Groningen, Groningen, The Netherlands

³GRIAC Research Institute, University Medical Center of Groningen, University of Groningen, Groningen, The Netherlands

⁴Department of Laboratory Medicine, Laboratory of Allergy and Pulmonary Diseases, University Medical Center of Groningen, University of Groningen, Groningen, The Netherlands

⁵Department of Allergology, University Medical Center of Groningen, University of Groningen, Groningen, The Netherlands

Background: There is need for an accurate diagnostic test in mastocytosis patients with wasp venom allergy (WVA) and monitoring of these patients during immunotherapy (IT). In this study, we aimed to evaluate sensitivity and specificity of the Basophil Activation Test (BAT) as a diagnostic and monitoring test in patients with mastocytosis and WVA.

Methods: Seventeen patients with mastocytosis and WVA and six mastocytosis patients without WVA were included. BAT was performed before the start of IT (first visit) and at 6 weeks (second visit) and 1 year (third visit), after reaching the maintenance dose. Of 17 patients included, 11 completed the third visit. In mastocytosis patients with WVA, dose-dependent wasp-venom induced upregulation of CD63 and CD203c expression on basophils was observed compared with mastocytosis patients without WVA. Serum specific IgE, IgG4, and tryptase levels were measured in all patients.

Results: BAT had a sensitivity of 87% and specificity of 100% in diagnosing WVA in mastocytosis patients. Basophil allergen threshold sensitivity with respect to CD63 and CD203c was significantly decreased in the second visit compared with the first visit and increased significantly in the third visit compared with the second visit. Specific IgE levels increased significantly in the second visit compared with first and decreased significantly in the third visit compared with the second. Specific IgG4 levels rose significantly in the second visit compared with the first and on the third visit compared with the second. Tryptase levels did not change significantly during the study.

Conclusions: BAT represents a diagnostic test with 100% specificity in allergic patients with mastocytosis and these patients are better to be monitored for a longer period during IT. © 2014 International Clinical Cytometry Society

Key words: basophil activation test; mastocytosis; wasp venom allergy; immunotherapy

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*Correspondence to: J.N.G. Oude Elberink, Department of Allergology, University Medical Center of Groningen, GRIAC Research Institute, University of Groningen, Groningen, The Netherlands.
E-mail: j.n.g.oude.elberink@umcg.nl and katayoonb@yahoo.com

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Insect venoms are common triggers of basophils and/or mast cells and cause severe systemic allergic reactions. The mechanisms could be immunologic or nonimmunologic (1,2). The diagnosis of wasp venom allergy (WVA) is based on the history of a reaction confirmed by skin test and/or specific IgE (sIgE) (3). However, in patients with severe reactions to Hymenoptera venom, an underlying systemic mastocytosis (SM) is frequently diagnosed (4). SM is a heterogeneous disorder characterized by clonal mast cell proliferation. The clinical spectrum varies from an indolent course with minimal symptoms to a progressive, potentially fatal disease. The World Health Organization (WHO) classifies seven types of mastocytosis. Indolent systemic mastocytosis (ISM) is the most common variant with the most favorable prognosis (5). Especially in ISM patients, the prevalence of Hymenoptera venom allergy is high (up to 47%) (6) and reactions are often severe. Diagnosis and management is usually problematic, especially in mastocytosis diagnosing insect venom allergy might be difficult as sIgE is low or even absent despite a history of anaphylaxis after an insect sting (7).

Venom immunotherapy (IT) is the only curative treatment with 90% success in WVA (8,9), but in WVA patients with mastocytosis, there are discussions about its efficacy. IT in mastocytosis patients has to be continued lifelong (7). It would be helpful if we can have an in vitro test to show the efficacy of allergen IT especially in WVA patients with SM.

The role of basophils in allergic immune responses and the reports of their alterations during IT, make them candidates in order to diagnose (10) and monitor allergic patients (11). Basophil Activation Tests (BATs) are in clinical use in WVA for a few years but the results about their efficacy and clinical value are inconsistent (3,12,13). Assessment of basophil allergen threshold sensitivity has been proposed as a promising tool to monitor basophil sensitivity in response to allergens (14–17). Of all markers studied, CD63 and CD203c have been more studied and have shown to be valuable in diagnosis and monitoring of allergic patients (18–20).

In this study, we firstly aimed to evaluate venom-induced CD63 and CD203c expression on basophils in order to determine the sensitivity and specificity of BAT

in patients with ISM with or without WVA. We also aimed to evaluate the alterations in BAT during a 1-year course of semi-rush IT by means of assessing allergen threshold sensitivity and reactivity.

METHODS

Seventeen patients suffering from ISM with a history of severe anaphylaxis to wasp stings (7 females and 10 males) and six ISM patients without WVA (all females) participated in this study. ISM was diagnosed based on WHO criteria (21,22). WVA was diagnosed based on history (grade II-IV SAR classified according to Mueller) (23) and proof of sensitization to wasp in intradermal skin testing. Intradermal skin testing was performed in all patients with inconclusive serologic sIgE measurements. Increasing concentrations of 0.03 ml Pharmedgen wasp (ALK-Abelló) ranging from 0.001 to 1 µg/ml were injected intradermally with a read out after 15 min. The skin test was considered positive if the wheal of the venom compared with wheal of the injected histamine [Histamine Equivalent IntraCutaneously (HEIC)] was at least 0.5. Absence of sensitization to wasp, based on history and negative intradermal skin test was the criteria to include ISM patients without WVA.

Demographic and disease related data of patients are included in Table 1. There were no significant differences between ISM patients with or without WVA regarding age and total IgE, but WVA patients had significantly higher levels of sIgE for wasp ($P=0.001$) and significantly lower levels for tryptase ($P=0.03$).

The study protocol was approved by the ethics board of University Medical Center Groningen (UMCG) and all patients had written consents for participation in the study.

A semi-rush schedule of IT was carried out in ISM patients with WVA. Rising doses of wasp venom (Pharmedgen-Wasp-Alutard, ALK-Abelló, Hørsholm, Denmark) were injected in the upper arm. On the first day, 0.0001–10 µg of wasp venom was injected at 30-min intervals, continuing with increasing weekly injections of 10 to 100 µg injections. Maintenance doses of 100 µg were injected every 6 weeks during 1 year. Blood was drawn before allergen injection at visits 1 (before the start of IT), 2 and 3 (after 6 weeks and after 1 year of

Table 1
Demographic and Disease-Related Data of the Participants

	Mastocytosis patients without WVA (N = 6)	Mastocytosis patients with WVA		
		Before IT (N = 17)	6 Weeks after reaching maintenance dose (N = 17)	1 Year after reaching maintenance dose (N = 11)
Female/male ratio	6/0	7/10	7/10	6/5
Age (yrs)	51 (27–68)	54 (36–73)	54 (36–73)	56 (39–67)
Tryptase level (µg/l)	47.2 (14.3–145)	28.8 (7.7–46)	27.2 (14.0–37.1)	27.6 (7.3–49)
Total IgE (kU/l)	22.7 (3.8–173.0)	28.0 (5.57–268)	29.1 (5.31–321)	19.5 (5.92–56)
Wasp specific IgE (kU/l)	0.005 (0–0.04)	0.57 (0–7.74)	1.49 (0.09–27.1)	0.44 (0.28–2.1)
Wasp specific IgG4 (mg/l)	NM	0.28 (0.04–8.44)	3.96 (0.1–24.2)	10.9 (5.06–24.4)

The results are presented as median (range).

NM: not measured.

reaching the maintenance dose, respectively). Of 17 patients who started IT, 11 patients continued IT for 1 year after maintenance dose.

Heparinized whole blood (100 μ l) was incubated with various concentrations of standardized wasp venom (0.5–5,000 ng/ml final concentration, ALK-Abelló) for 30 min at 37°C. Antihuman IgE antibody (10 μ g/ml, BD Pharmingen) and RPMI were used as positive and negative controls, respectively. The reaction was stopped by chilling on ice and fluorescence-conjugated antibodies (Anti-CD63-FITC, Anti-CD45-PerCP (both from Becton, Dickinson and Company, NJ), Anti-CD203c-APC and Anti-IgE-PE (both from Miltenyi Biotec, Bergisch Gladbach, Germany)) were added to the tubes for 30 min at 4°C. Red blood cells were lysed using lysing solution (Becton, Dickinson), cells were washed and fluorescence was measured on a FACS Calibur (Becton, Dickinson). Flowcytometric data was analyzed using Winlist software (Verity Software House, Topsham). Basophils were detected based on forward and side scatter and expression of CD45 and IgE. Quadrants were set based on the fluorescence of unstimulated cells (negative control) (Fig. 1). The basophils of all participants showed clear positive results when stimulated with anti-IgE antibodies as positive controls.

The change in threshold sensitivity was evaluated by basophil CD63 and CD203c response at submaximal concentrations (17,24,25). LC50 (log 10 of the allergen concentration which causes 50% basophil activation) was calculated after transformation and normalization of data and was compared between different visits. Higher values of LC50 indicated less basophil sensitivity.

Serum levels of total IgE (tIgE), sIgE to wasp, venom sIgG4, and tryptase were measured with fluorescence enzyme immunoassay (CAP-FEIA system, Phadia, Uppsala, Sweden).

SPSS 18 was used for data analysis. Wilcoxon signed rank test was used to compare parameters in two visits. Quantitative variables were presented as median (range). *P* values <0.05 were considered significant. The discrim-

inative value of BAT was evaluated by constructing receiver operating characteristic (ROC) curves.

RESULTS

BAT in the Diagnosis of WVA in Mastocytosis Patients

Median percentages of CD63 expression in wasp venom concentrations of 0.5 to 5,000 ng/ml in mastocytosis patients with and without WVA ranged from 0.4 to 36.05% and 0.39 to 0.82%, respectively. Median percentages of CD203c expression in wasp venom concentrations ranging from 0.5 to 5,000 ng/ml in mastocytosis patients with and without WVA was 1.49 to 74.33% and 0.22 to 0.51%, respectively.

In WVA patients with mastocytosis, dose-related up-regulation of CD63 and CD203c expression was observed, while in mastocytosis patients without WVA, the percentages of CD63 and CD203c expression did not increase at higher wasp venom doses (Fig. 2). Based on ROC curves, venom concentrations of 50 to 5,000 ng/ml for CD63 and 0.5–5,000 ng/ml for CD203c could significantly discriminate between ISM patients with and without WVA (Table 2).

BAT in Monitoring of WVA in Mastocytosis Patients Under IT

Before immunotherapy and 6 weeks after the maintenance dose

In wasp venom concentrations of 5 to 50 ng/ml, the percentage of basophils expressing CD63 significantly decreased after receiving the maintenance dose compared with the time point before the start of IT (in 5 ng/ml: from 2.6 (0–35) to 0.19 (0–2), *P* = 0.003; in 50 ng/ml: from 3.8 (0.38–62.0) to 2.0 (0–46.0), *P* = 0.005) (Fig. 3).

CD203c expression decreased significantly after receiving the maintenance dose compared with the time point before the start of IT in wasp venom concentrations of 0.5 ng/ml (from 1.49 (0.21–20.0) to 0.59 (0–4.0),

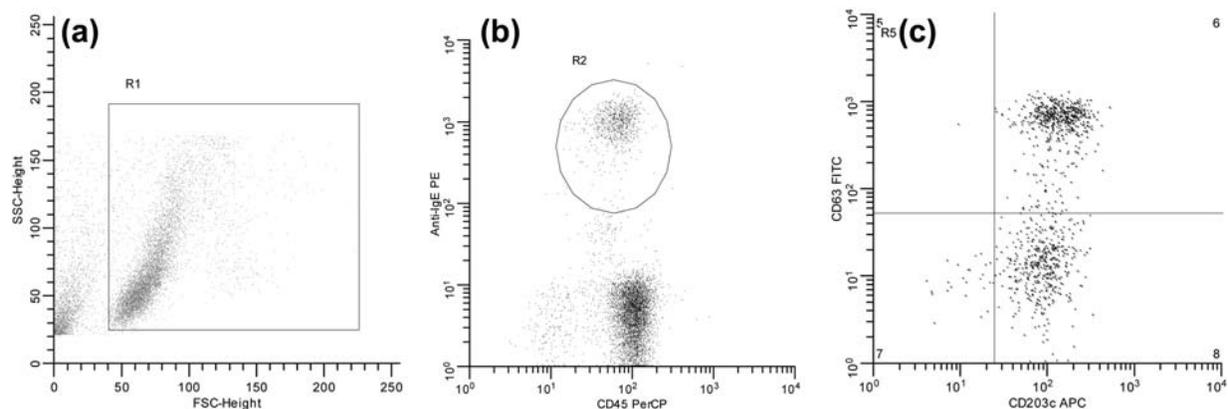


FIG. 1. Flowcytometric analysis of data of one representative patient. The gating strategy is shown. **a.** Viable cells were gated based on forward and side scatter. **b.** Subsequently, basophils were gated as CD45+Anti-IgE+ cells. **c.** Dots show CD63 and CD203c expression with optimal wasp venom concentration.

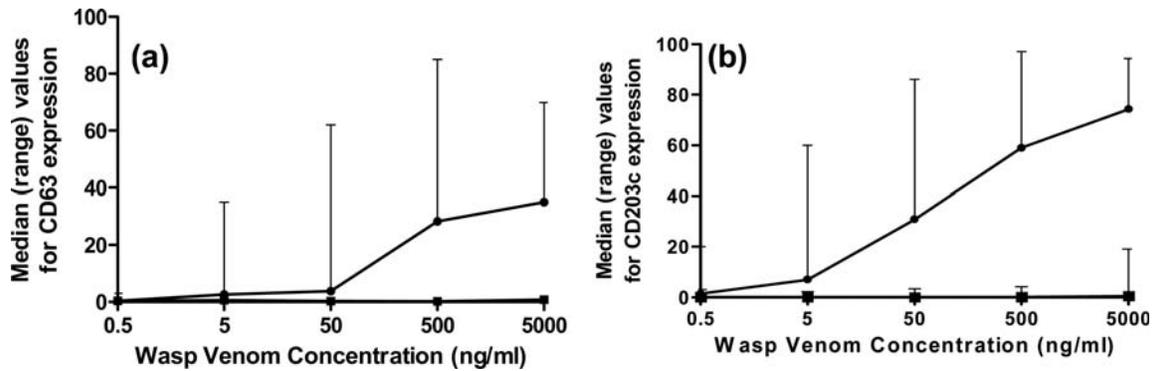


FIG. 2. Median (range) percentages of CD63+ and CD203c+ basophils in response to wasp venom in mastocytosis patients with (circles) and without WVA (squares). a. CD63 expression. b. CD203c expression.

$P = 0.02$), 5 ng/ml (from 7.0 (0–60.0) to 0.5 (0–32.0), $P = 0.003$), 50 ng/ml (from 30.8 (0.5–86.0) to 3.5 (0–85.0), $P = 0.01$) and 500 ng/ml (from 59.0 (0.5–97.0) to 34.5 (1.0–93.0), $P = 0.04$) (Fig. 3).

Maximum percentage of cells expressing CD63 or CD203c did not change significantly between the first two visits.

After reaching the maintenance dose (from 6 weeks to 1 year)

CD63 expression increased significantly after 1 year of reaching the maintenance dose compared with 6 weeks after the maintenance dose in wasp venom concentrations of 5 ng/ml (from 1 (0–3.0) to 1.0 (0–7.0), $P = 0.02$), 50 ng/ml (from 2.0 (0–46.0) to 3.0 (0–53.0), $P = 0.01$) and 500 ng/ml (from 14.4 (0–63.0) to 27.0 (12.0–85.0), $P = 0.01$) (Fig. 3a). CD203c expression also increased significantly 1 year after reaching the maintenance dose compared with 6 weeks after that in wasp venom concentrations of 0.5 ng/ml (from 0.59 (0–4.0) to 3.0 (0–6.0), $P = 0.04$), 5 ng/ml (from 0.5 (0–32.0) to 3.0 (0–16.0), $P = 0.04$) and 50 ng/ml (from 3.5 (0–85.0) to 18.7 (1.0–74.0), $P = 0.02$) (Fig. 3). Maximum percentage of basophils expressing CD63 or CD203 did not change significantly between the second and third visits.

A shift to the right was observed in dose-response curves in the second visit (6 weeks after the mainte-

nance dose) compared with the first visit (before IT) for CD63. LC50 for CD63 was significantly increased in the second visit compared with the first visit (1.38 ± 0.36 in the first visit compared with 3.33 ± 1.5 in the second visit, $P = 0.01$). The third visit curve was not significantly different from first or second visit curves and LC50 for CD63 of the third visit was calculated to be 2.51 ± 0.29 (Fig. 4). LC50 for CD203c was not significantly different between the three visits (LC50 for first visit: 1.47 ± 0.66 , for the second visit: 2.49 ± 0.57 , the third visit: 2.24 ± 0.21).

slgE, slgG4, and Tryptase levels

slgE levels increased significantly in the second visit compared with the first visit and decreased significantly in the third visit compared with the second one. There were no significant differences between slgE levels of the first and the third visit (Fig. 5 and Table 1). On the other hand, slgG4 levels increased in the second visit compared with the first visit and further increased in the third visit (Fig. 5 and Table 1). Tryptase levels did not change significantly in the three visits.

DISCUSSION

Cellular tests such as Histamine release tests (HRT) and BAT seem to improve the diagnosis of WVA

Table 2
Data of ROC Curves for Wasp Venom Concentrations of 50–5,000 ng/ml for CD63 and 0.5–5,000 ng/ml for CD203c in Mastocytosis Patients with ($n = 17$) and without ($n = 6$) WVA

	AUC	P value	Threshold value	Sensitivity %	Specificity %
CD63 (ng/ml)					
50	0.89	0.005	1.53	68	100
500	0.94	0.002	1.11	87	100
5,000	0.91	0.003	2.39	87	100
CD203c (ng/ml)					
0.5	0.82	0.02	4.0	31	100
5	0.83	0.01	2.45	62	100
50	0.94	0.002	4.16	75	100
500	0.96	0.001	5.58	87	100
5,000	0.95	0.001	24.66	81	100

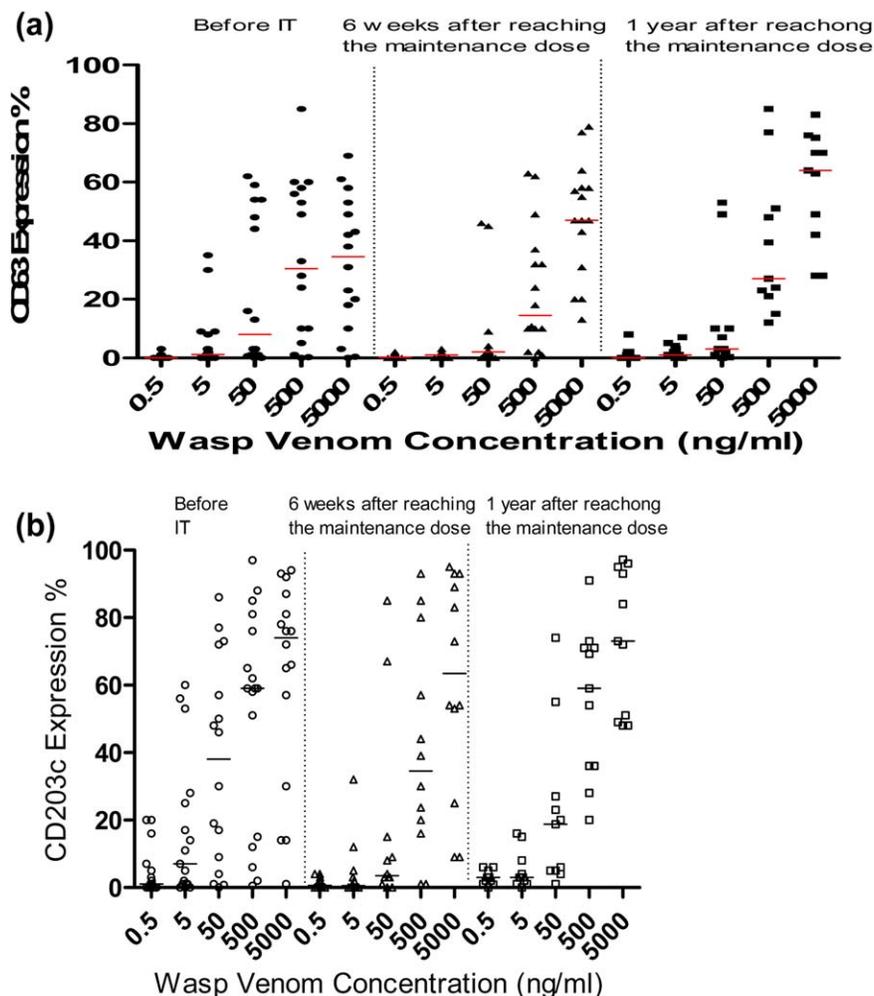


FIG. 3. Percentage of CD63 and CD203c expression on basophils after activation with different wasp venom concentrations (0.5–5,000 ng/ml) at first to third visits (a) CD63 expression and (b) CD203c expression at first, second, and third visits. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

(10,25–28). BAT has been compared with HRT and the results have shown a possible similar mechanism for these two tests (26).

Mastocytosis is a risk factor for severe systemic reactions in WVA (29). In this study, we could show that the sensitivity and specificity of BAT in mastocytosis patients

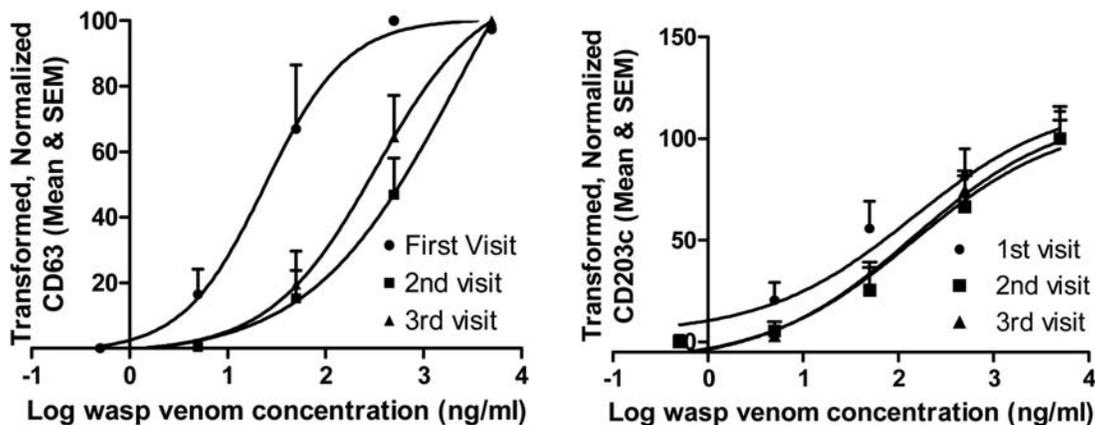


FIG. 4. Dose–response curves of CD63 and CD203c expressions in response to wasp venom. Normalized mean and SEM values of CD63 and CD203 are shown respectively.

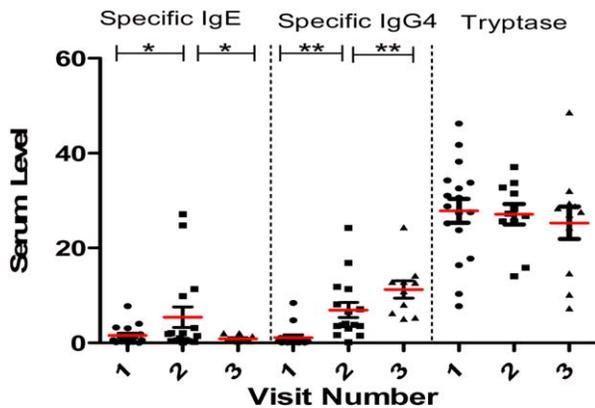


FIG. 5. Serum levels of sIgE (kU/l), sIgG4 (mg/l), and tryptase (μ g/l) before immunotherapy (first visit), after 6 weeks (second visit) and 1 year of reaching the maintenance dose (third visit). Mean \pm SEM values are plotted in the graph. * $P < 0.05$; ** $P < 0.005$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

for the diagnosis of WVA were 87% and 100%, respectively. It was similar to previous studies in WVA patients without mastocytosis, that reported sensitivity and specificity of BAT using CD63 was 85 to 100% and 83 to 100%, and using CD203c was 89 to 100% and 89 to 92%, respectively (18). ROC curves showed the proper cut off values and venom concentrations that can be used to diagnose WVA in mastocytosis patients. The optimal venom concentration was 500 ng/ml, both for CD63 and CD203c. However, our selection criteria based on positive skin tests could have influenced these results because of the good correlation between skin tests and sIgE and BAT.

In the study by Korosec et al., SPT and sIgE were negative in 4% of patients with a history of systemic reactions to stings. They could show that BAT was a reliable test in these patients complementary to intradermal tests to increase diagnostic sensitivity, irrespective of the period between reaction and the test (30). Ebo et al. also found out that BAT can be an additional diagnostic test in difficult cases with negative sIgE or skin tests (31).

However, in a study by Bonadonna et al., BAT had no additional value in Hymenoptera venom allergic patients with systemic mastocytosis and negative skin tests (32). All these studies (30–32) have interpreted BAT as being either positive or negative, which is in contrast to our study in which we evaluated the basophil allergen threshold sensitivity by examining several venom concentrations in the BAT.

IT is a highly effective treatment to reduce severe, life threatening reactions in WVA patients (29). It leads to complete or partial protection in WVA patients, but long term effects are not truly revealed (33). Increase in allergen specific immunoglobulins which mainly, but not only, contain IgG4 and IgA and a decrease in the ratio of free allergen specific immunoglobulin to allergen sIgE bound to mast cells and basophils could account for the effects of IT (19). Furthermore, decrease in peripheral

blood basophil numbers, their activation status, expression of surface antigens and changes in cytokines and chemokines, early in the phase of IT, were reported previously (34–36). In a study by Nullens et al, a new method to evaluate histamine content of basophils showed that 6 months IT reduced basophil numbers and also their histamine content and release in allergic patients (37).

Recent studies propose that the use of basophil allergen threshold sensitivity in BAT rather than scoring a positive or negative result at a set allergen dose is useful in determining patient's allergen sensitivity (14,15,17) and that this allergen threshold sensitivity decreases in some patients during the course of IT (38). Ebo et al., have demonstrated decreased basophil responsiveness in WVA patients after 6 months of IT and also cross-sectionally in patients receiving IT for 3 years, as a decision tool to discontinue IT (25). Kucera et al. also showed that BAT could represent a useful tool to determine the outcome of IT after approximately 4 years in venom allergy (11). BAT is considered as a marker for monitoring of IT (25), but maximal response of basophils is not an informative measure of clinical response of the patients (14,15,17). Use of dose-response curves was recommended for follow up of an individual during treatment, like IT (39). Despite all available data on IT in WVA patients, little is known about IT in mastocytosis patients with WVA. There is still no consensus about optimal maintenance dose, intervals between injections, duration of treatment and management of side effects (7). Gonzalez-de-Olano et al., retrospectively, evaluated BAT by means of CD63 level expressions on basophils in WVA patients with mastocytosis. However, their control group consisted of WVA patients without mastocytosis. Their diagnosis was based on sIgE levels higher or equal to 0.35 as cut off values. Their BATs were reported as positive or negative. They reported BAT as a tool for diagnosis and prediction of the outcome of IT in mastocytosis patients (40).

Our study, similar to some recent studies (16,19), showed that basophil sensitivity is a good marker for monitoring of IT in mastocytosis patients. We compared percent activation of CD63 and CD203c in four submaximal concentrations of wasp venom and also calculated the logarithm of the concentration of allergen that elicited a half-maximal response (LC50). Reactivity of basophils was also calculated by their maximal response. In this study, we could show that basophils were less sensitive to wasp venom after reaching the maintenance dose and the LC50 increased by more than 1 unit. This was in accordance with the study of Mikkelsen et al. on WVA that showed more than 1 unit LC50 increase in WVA patients who had reached maintenance dose compared with the time before IT. They associated this increase in LC50 with a protective effect of plasma attributable to immunoglobulins (19). However, in our study, basophil sensitivity did not change significantly after 1 year of IT compared with before the start of immunotherapy and 6 weeks after immunotherapy. This

could be due to larger intervals between injections after receiving the maintenance dose or the different mechanisms of tolerance in long-term IT versus short-term IT (41). Certainly, for considering BAT as a monitoring tool in IT, more studies are needed with larger sample sizes and longer follow-up. The significant increase in serum IgG4 after a year of IT was not associated with basophil sensitivity. We observed an increase in sIgE levels just after reaching the maintenance dose which returned to the baseline after 1 year of IT. In the future studies, a comparative analysis on washed cells might be a better approach to evaluate changes in antibody concentrations.

In our analysis, similar to the study by Mikkelsen et al. (19), we both used CD63 and CD203c for diagnosis and monitoring of WVA patients. The results were almost identical and comparable for both markers. It can be inferred that although these two markers are different, they might be regulated similarly when basophils are activated. Recent studies have focused on identifying new markers such as p38 mitogen-activated protein kinase (MAPK) in the diagnosis of WVA (42) and signal transducer and activator of transcription (STAT)5 in order to elucidate possible mechanisms of basophil degranulation (43).

In conclusion, performing BAT in complete blood represents a diagnostic test with 100% specificity as well as a useful monitoring tool for therapy in allergic patients with ISM which reflects the response of the cells in the context of immunoglobulins present in the blood.

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