

Original Article

Basophilic Histamine Content and Release During Venom Immunotherapy: Insights by Flow Cytometry

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Background: Despite the efficiency of venom immunotherapy, the effects on basophils and mast cells remain incompletely understood and probably vary according to the treatment phase.

Objectives: To study the effect of build-up and maintenance venom immunotherapy on individual basophils.

Methods: Intracellular histamine and its release was analyzed flow cytometrically by a new enzyme-affinity method using diamine oxidase conjugated to laser-excitable fluorochromes.

Phenotyping of cells included flow cytometric quantification of CD63 and CD203c. Analyses of basophil activation experiments were performed before the start of treatment, after build-up therapy and during maintenance therapy.

Results: Before the start of therapy, patients demonstrated significantly higher numbers of basophils when compared with stung control individuals. At the end of build-up therapy a decrease of basophil numbers was observed, whereas during maintenance therapy basophil counts returned to pretreatment values. Before the start of therapy, the intracellular histamine content per cell in patients was significantly higher when compared with stung control individuals. During maintenance therapy intracellular histamine content decreased to values observed in stung control individuals. In addition, maintenance therapy lowered the net release of histamine per cell in response to optimal stimulation with wasp venom.

Conclusions: We introduce a novel technique that enables to assess the effects of venom immunotherapy on basophils. This new technique may help to monitor treatment effects in individual patients and could aid in the development of more efficient and better tolerated immunotherapy protocols. © 2013 International Clinical Cytometry Society

Key terms: basophil; histamine; allergen immunotherapies; flow cytometry

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IgE-mediated venom allergy constitutes an important health issue with significant impact on patients' quality of life, morbidity, and mortality (1,2). Therefore, venom-specific immunotherapy (VIT) should be considered in all patients who suffer from venom-induced anaphylaxis (3,4). Despite the rapid and high efficiency of VIT (5,6), the underlying mechanisms contributing to clinical tolerance remain incompletely understood and probably vary

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according to the treatment phase. Currently, most of our knowledge on tolerogenic effects of VIT relates to T cell phenomena and the production of blocking antibodies during maintenance VIT (7,8). In contrast, a better understanding of the mechanisms that govern early tolerance phenomena could greatly help to optimize therapy, to reduce or avoid adverse events, and to identify biomarkers enabling efficacy monitoring. The prevailing hypothesis is that the repetitive administration of incrementing doses of allergen during the build-up phase induces a transient depletion/exhaustion or inhibited release of mediators from basophils and mast cells. Several authors showed that the allergen-induced release of histamine and/or leukotrienes by purified basophils is reduced during or after build-up treatment (9–13). This is likely caused by a continuous release of mediators below the threshold of anaphylaxis (14), rather than a decrease in the synthesis of mediators (15). Interestingly, a continuous release of histamine (e.g., by piecemeal degranulation) could exert an autocrine inhibitory function through the histamine 2 receptor. In addition,

early clinical tolerance might also result from reduced numbers of basophils (16,17).

Recently, we developed a flow cytometric technique enabling simultaneous analysis of histamine release and surface markers of individual basophils in whole blood. We demonstrated that this technique captures data that cannot be obtained by traditional mediator release assays (18). In contrast to traditional mediator release tests, our method does not require homogeneous cell populations and our results do not represent an average of purified leukocytes as the technique allows for histamine measurements on a single cell level.

In this study, we used our assay to investigate the effects of early and maintenance VIT on the content and release of histamine by individual basophils kept in their natural environment.

METHODS

Patients and control individuals

The demographic data of patients and control individuals are displayed in Table 1. We applied VIT (ALK-Abello, A/S, Denmark) to 23 adults with wasp venom allergy starting with a 3 day build-up phase and subsequent monthly injections of a maintenance dose of 100 µg yellow jacket (YJ) venom (19). All patients had a history of a systemic anaphylactic reaction to wasp (*Vespa vulgaris*) venom evaluated by a standardized questionnaire, sensitization to wasp venom as documented by a positive sIgE and/or skin test, and were responsive in the traditional CD63-based basophil activation test. Figure 1 displays the timetable of visits. Blood was obtained immediately before the start of treatment and within the hour after the last injection of the build-up phase. The build-up phase consisted of a semirush protocol, during which patients received increasing dosages of subcutaneously injected purified wasp venom, reaching the highest dose of 100 µg after 3 days of therapy. In 14 patients blood was also taken after 6 months of maintenance therapy, immediately before the maintenance injection. Ten stung control individuals were also included. Control patients were selected based on anamnesis of having been stung at least once in their lifetime without suffering an allergic reaction and demonstrating a negative sIgE to wasp venom (<0.35 kUA/L). All patients and control individuals signed a written

Table 1
Demographics and Characteristics of Patients and Stung Control Individuals

	Venom immunotherapy—patients		Stung controls
	Day 0/Day 3	Month 6	
Number (n)	23	14	10
Male/female	13/10	10/4	5/5
Median age	49	48.5	48
(range)	(9–74)	(9–74)	(16–63)
Total IgE (kU/L)	143	ND	ND
(range)	(11–1101)		
Wasp IgE (kUA/L)	3.56	ND	<0.35
(range)	(<0.35–58.7)		
Serum tryptase level (µg/L)	6.75	ND	ND
(range)	(1.3–10.1)		
Percentage of positive skin test for wasp	20/23 (87%)	ND	ND
Severity of reaction	Systemic reaction following a wasp sting: ≥ grade 2 according to Mueller's classification		ND

ND, Not determined.

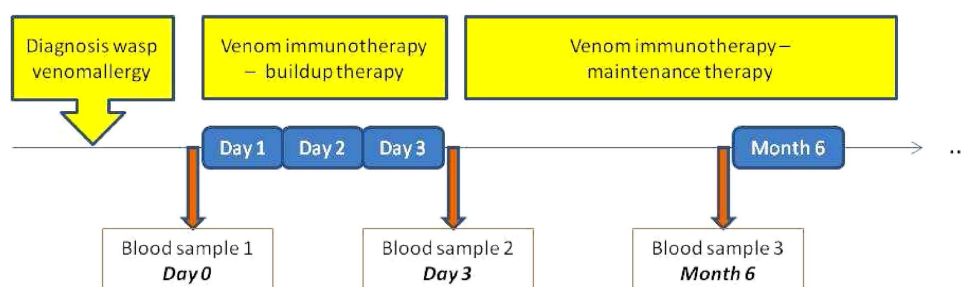


Fig. 1. Time line of therapies and drawing of blood samples. [Color figure can be viewed in the online issue which is available at wileyonlinelibrary.com.]

informed consent approved by the local ethical committee.

Basophil Counts

White blood cells (WBC) were counted flow cytometrically by reversed pipetting 100 μ L heparinized blood, 500 μ L standardized beads (Flow Cytometry Absolute Counts Standard, Bangs Laboratories, Fishers, IN), and 10 μ L CD45 FITC (BD Biosciences, Erembodegem, Belgium), incubating for 20 min at room temperature and lysed with FACSlyse (BD Biosciences). The WBCs were gated out on side scatter and CD45 positivity and the number of WBC was determined with reference to the counted beads. Basophils were counted flow cytometrically using CD45-FITC, CD203c-APC (clone NP4D6 Biologend, San Diego, CA) and monoclonal anti-human IgE (clone GE-1, Sigma Aldrich GmBH, Steinheim, Germany) labeled with Alexa Fluor 405 (Molecular Probes, Invitrogen, Paisley, UK) in 100 μ L whole blood. Absolute basophil counts were expressed as number of cells per mL.

Basophil Activation

Endotoxin-free heparinized whole-blood samples were obtained from healthy donors and venom allergic patients. Aliquots of 200 μ L whole blood were challenged at 37°C with 200 μ L anti-IgE (10 μ g/mL; clone G7-18, BD Biosciences) as positive control, 200 μ L activation buffer [Hank's balanced salt solution (Invitrogen, Paisley, UK)] with 20 μ M HEPES and 7.5% NaHCO₃, pH 7.4, without IL-3) as a negative control, or wasp allergen [ALK-Abello, A/S, Denmark 1 μ g/mL: for dose-finding see (20)] for 20 minutes. Reactions were stopped by immediately chilling on ice and adding 1 mL ice-cooled PBS with 10 mmol/L EDTA. Samples were then centrifuged for 5 min (4°C, 200 g).

Immunophenotyping

To determine the number of activated basophils, cells were stained with 20 μ L of monoclonal anti-human IgE (clone GE-1, Sigma Aldrich GmBH, Steinheim, Germany) labeled with Alexa Fluor 405 (Molecular Probes, Invitrogen, Paisley, UK) and 10 μ L of monoclonal anti-human CD63-FITC (clone H5C6, BD Biosciences), 10 μ L CD203c-APC (clone NP4D6 Biologend, San Diego, CA) for 20 min on ice.

Cell Fixation and Permeabilization for Intracellular Staining

Cells labeled for IgE, CD63, and CD203c were fixed with 2 mL Phosflow Lyse/Fix Buffer (BD biosciences, Erembodegem, Belgium) for 20 min at 37°C. Cells were then washed with and resuspended in PBS with 0.1% Triton-X-100 (PBS-TX, pH = 7.4). To stain intracellular histamine, 10 μ L Phycoerythrin (PE) labeled diamine oxidase (DAO, BD Biosciences) was added and incubated at 37°C for 45 min. Cells were washed with 0.3 mL PBS and resuspended in PBS with 0.1% sodium azide. Control settings with and without permeabilization and addition of unlabeled DAO to measure specific staining were used.

Flow Cytometric Analysis

Flow cytometric analysis was performed on a FACS-Canto II flow cytometer (BD Immunocytometry Systems, San Jose, CA) as described by Ebo et al. (18). Correct compensation setting for the fluorochromes used was performed using BD CompBeads (BD Biosciences). The percentage of activated basophils was determined to ascertain whether patients were responsive to the traditional CD63-based basophil activation test (inclusion criteria for patients). Fluorescence minus one (FMO) and DAO staining without permeabilization were used to set a marker between DAO positive and negative cells. Flow cytometric characterization of basophils relied on a combination of two density plots, that is, side scatter (SSC) and anti-IgE and anti-IgE and CD203c. At least 1000 basophils were counted and evaluated. The IgE intensity on basophils was not different after build-up or maintenance therapy, compared with pretreatment values.

Standardization

Standardization of intracellular histamine content was performed using standardized fluorospheres (SPHERO Ultra Rainbow Calibration particles, Spherotech, Lake Forest, IL) as described by the manufacturer. Beads were measured with the same settings as for the cell analysis and the median was correlated to the fluorescence intensity of the beads using linear regression. Results were expressed as median fluorescence intensity (MdfI) per cell and percentage positive/negative cells. We determined three types of activated cells, that is, CD63⁻CD203c^{bright+}, CD63^{dim+}CD203c^{bright+}, and finally CD63^{bright+}CD203c^{bright+} cells (see Fig. 4), according to results previously published by our research group (18). CD203c^{bright+}CD63^{bright+} cells demonstrate a clear histamine release, whereas CD203c^{bright+}CD63^{dim+} cells show less histamine release. We therefore calculated the median histamine release as the difference between MdfI per cell in nondegranulated cells (CD63⁻ and CD203c^{dim+}) minus the MFI per cell in degranulated cells (CD63^{bright+} and CD203c^{bright+}).

Statistics

Analysis of data and calculations was performed using IBM SPSS Statistics version 20.0 (IBM, Chicago, IL). All results were expressed as median (range). The Wilcoxon signed rank test was used to compare basophil counts and intracellular histamine content of allergic patients at day 0, day 3, and month 6. The Mann-Whitney U test was used to compare the data between patients and stung control individuals.

RESULTS

Characteristics of Patients and Stung Control Individuals

The demographics and characteristics of the patients, including results of total IgE, sIgE, and skin prick testing, and gender and age-matched stung control individuals are displayed in Table 1. Baseline serum tryptase

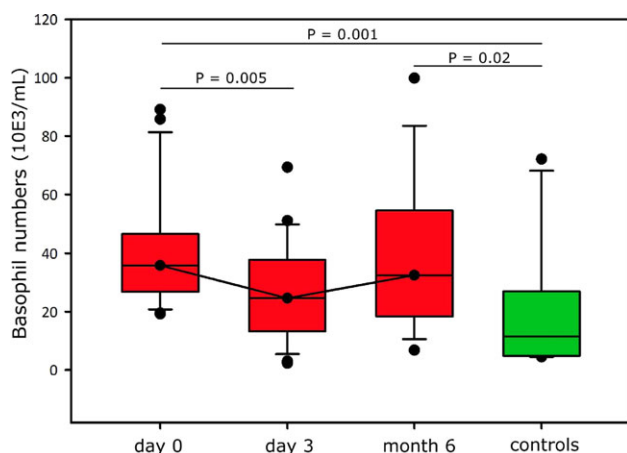


Fig. 2. Basophil numbers are increased in wasp venom-allergic patients, and drop during the first days of specific immunotherapy. Absolute counts of basophils ($\times 10^3/\text{mL}$) in wasp venom-allergic patients (red) before therapy (day 0), after the venom immunotherapy (VIT) build-up phase (day 3) and during VIT maintenance treatment (month 6) when compared with stung control individuals (controls, green).

level, determined immediately before initiation of the build-up therapy, was normal (i.e., $< 11.4 \mu\text{g/L}$) in all patients, thus excluding mastocytosis. All patients received VIT because of their history of wasp venom allergy and only when unequivocally testing positive for sIgE and/or wasp venom skin prick testing. Only patients reactive in a traditional CD63-based BAT were assessed for basophil responsiveness during VIT.

Basophil Counts

Basophil numbers were found to be increased in wasp venom-allergic patients, and dropped during the first days of specific immunotherapy (Fig. 2). In patients, basophil numbers (median) were markedly, that is about threefold, higher when compared with stung control individuals ($35.80 \times 10^3/\text{mL}$ vs. $11.55 \times 10^3/\text{mL}$, $P = 0.001$, Fig. 2). Basophil numbers in patients dropped significantly, from $35.80 \times 10^3/\text{mL}$ to $24.60 \times 10^3/\text{mL}$ ($P < 0.005$) during venom immunotherapy build-up treatment. During maintenance VIT, basophil numbers returned to pretreatment baseline values to $32.45 \times 10^3/\text{mL}$.

Basophil Intracellular Histamine Content

Basophil histamine content was also found to be higher in wasp venom allergic patients when compared with control subjects and to drop during VIT. Patients' basophils contained ~ 2.5 times the histamine found in the basophils of control subjects (409 vs. 156 MdFI/cell; $P = 0.01$; Fig. 3). Patients' intracellular basophil histamine content dropped to about half their pretreatment baseline levels after 6 months of VIT to 232.50 MdFI/cell ($P = 0.03$, Figs. 3 and 4).

VIT also reduced allergen-induced MHR/cell in wasp allergic patients (Figs. 4 and 5). A significant lower

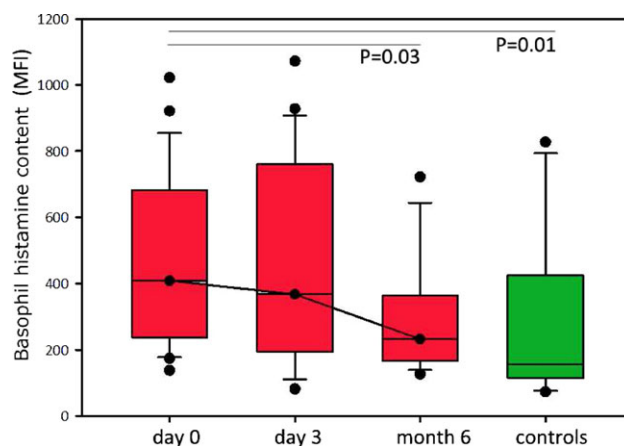


Fig. 3. Basophil histamine content is increased in wasp allergic patients, and drops after initiation of specific immunotherapy. Median basophil histamine content per cell in wasp venom-allergic patients (red) before therapy (day 0), after the venom immunotherapy (VIT) build-up phase (day 3) and during VIT maintenance treatment (month 6) when compared with stung control individuals (controls, green). MdFI = median fluorescence intensity.

MHR/cell was observed after 6 months of VIT (304 vs. 176 MHR/cell; $P < 0.05$).

DISCUSSION

To our knowledge this is the first study showing that VIT reduces the content and release of histamine by individual peripheral venous blood basophils in allergic patients. It appears these effects differ significantly depending on the treatment phase, which is in line with the different tolerance mechanisms proposed to be responsible for the protection provided during early and maintenance treatment (7,8).

At baseline, before the start of VIT, wasp venom allergic patients demonstrated significantly higher counts of peripheral blood basophils containing a basal mean histamine content that is 2.5-fold higher when compared with stung control individuals. These findings could help to explain the increased susceptibility of the patients to develop anaphylaxis and are in line with former observations that patients suffering from IgE-mediated conditions such as allergic rhinitis (21) or atopic dermatitis (22) exhibit an altered histamine metabolism.

Although evidence has been provided that the continuous administration of incrementing doses of allergen might induce transient tachyphylaxis of effector cells (9–13), the exact tolerogenic mechanisms of early VIT remain incompletely understood. Our experiments confirm that VIT build-up significantly lowers the number of circulating basophils to values observed in stung control individuals. These findings parallel the observations of others (16,17) and fit in a mechanistic model in which an increased deletion by apoptosis of circulating basophils is considered as a potential explanation of early tolerance (17). In contrast, we show that build-up VIT has no significant effect on the intracellular

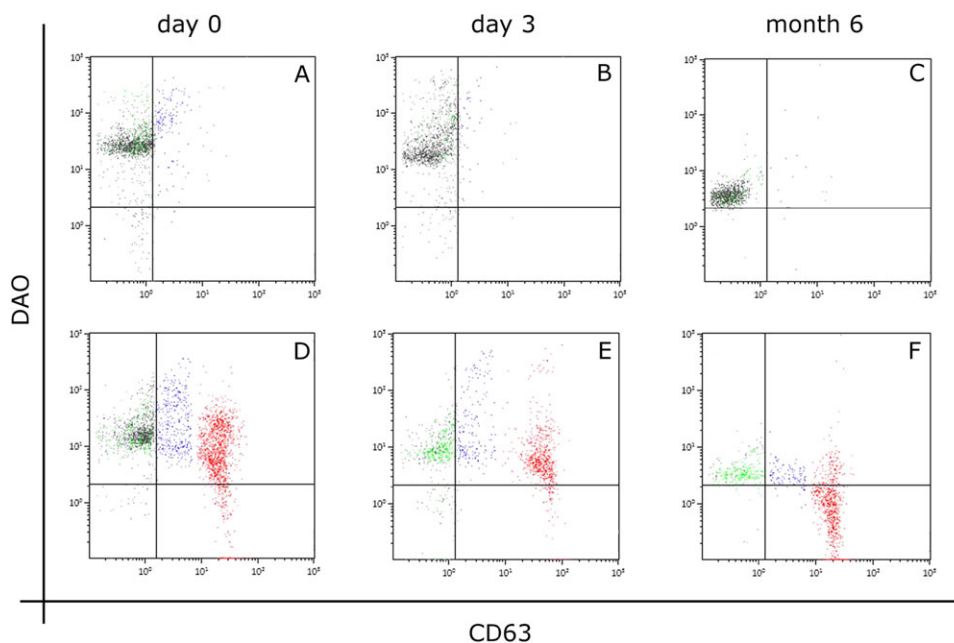


FIG. 4. Representative sample of histamine content of resting basophils (top panels) and release after stimulation of cells with wasp venom (bottom panels) before venom immunotherapy (day 0), at the end of a build-up treatment (day 3) and during maintenance therapy (month 6). The threshold of intracellular histamine content is set at the lowest level observed at month 6 of maintenance immunotherapy. Note the decrease over time in resting cells and decreased median histamine release (MHR)/cell in response to wasp venom (1 $\mu\text{g/mL}$). DAO: Diamine-oxidase. Black dots denote resting cells. Activated cells are divided in three populations, that is, $\text{CD63}^{\text{dim}}\text{-CD203}^{\text{bright+}}$ (green), $\text{CD63}^{\text{dim}}\text{-CD203}^{\text{bright+}}$ (blue), and finally $\text{CD63}^{\text{bright+}}\text{-CD203}^{\text{bright+}}$ (red) [see (18)].

histamine content of circulating basophils. This parallels the recent observations of Maintz et al.(15), who reported normal mRNA levels of L-histidine decarboxylase (the enzyme responsible for histamine synthesis) at the end of early VIT. Finally, our experiments confirm that build-up VIT does not affect the *in vitro* responsiveness of basophils (20,23–25) or the median release of histamine per cell. Thus, the effect of early VIT is likely to be related, at least partially, to a reduction of circulating basophils.

In contrast, as after 6 months of VIT, basophil numbers completely recovered and returned to pretreatment levels, it is clear that the effectiveness of maintenance therapy has to be sought in alternative mechanisms (7,8). A relevant finding in this context is our observation that during treatment, resting basophils demonstrate a progressive reduction of stored histamine. After 6 months of therapy mean intracellular histamine content is markedly reduced and comparable to values in stung control individuals. Furthermore, maintenance therapy was confirmed to suppress basophil responsiveness (20,23–26) and to significantly lower net release of histamine per cell in response to optimal stimulation with wasp venom compared to before the start of treatment. As comprehensively addressed elsewhere (7,8), the exact mechanisms that steer and govern the protective effects of VIT are complex and remain incompletely understood. However, it seems likely that our technique, which allows for a simultaneous assessment of activation markers, inhibitory receptors, signaling molecules, and

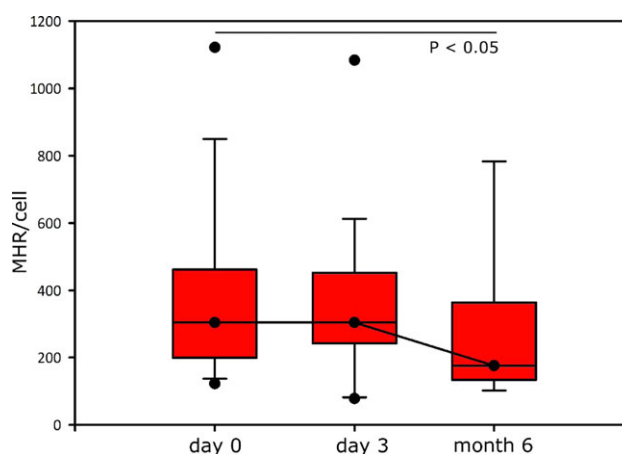


FIG. 5. Wasp allergic patients on maintenance specific immunotherapy exhibit reduced allergen-induced basophil histamine release. Median wasp venom-induced basophil histamine release in wasp venom-allergic patients before therapy (day 0), after the build-up phase of venom immunotherapy (VIT, day 3) and during VIT maintenance treatment (month 6). MHR = Median histamine release.

mediator release, can help to disentangle the early and late effects of VIT on basophils.

Taken together, we introduce a novel technique to study the effects of VIT on basophils. We show that patients with wasp venom allergy exhibit significantly higher numbers of circulating basophils with elevated histamine stores. Build-up VIT induces a transient decrease of basophil counts without significant effect on

individual basophil histamine content or release. In contrast, maintenance VIT is confirmed to lower the content and median release of histamine by basophils on stimulation with wasp venom allergen. Our technique and findings may help to monitor treatment effects in individual patients and could aid in the development of more efficient and better tolerated immunotherapy protocols.

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