

Original article

High sensitivity of basophils predicts side-effects in venom immunotherapy

Background: Systemic side-effects of venom immunotherapy (VIT) represent a considerable problem in the treatment of patients allergic to Hymenoptera venom. We examined the hypothesis whether basophil responsiveness might be connected with the adverse reactions to VIT.

Methods: Basophil surface expression of activation marker CD63 induced by different concentrations of honeybee and wasp venom (0.1 and 1 µg/ml) was measured by flow cytometry in 34 patients with history of systemic anaphylactic reactions to Hymenoptera sting just before rush honeybee or wasp VIT.

Results: Eleven of 34 patients had systemic anaphylactic reaction (Mueller grades I–III) and one patient a large local reaction to VIT. In those 12 patients, median percentage of activated basophils after stimulation with VIT-specific venom in concentration of 0.1 µg/ml was 99% (range: 17–195) of value reached with stimulation with 1 µg/ml. Side-effects occurred in all patients with 0.1/1 ratios over 92% (eight of 12). In contrast, in 22 patients with no side-effects, the median 0.1/1 ratio was 25% (range: 2–92). These concentration-dependent activation ratios were significantly different between the groups with and without side reactions ($P < 0.0001$). We also show significant positive correlation of the occurrence/clinical grade of the side-effects with individual ratios of CD63 basophil response ($r = 0.73$, $P < 0.0001$).

Conclusion: The results suggest that increased basophil sensitivity to allergen-specific *in vitro* stimulation is significantly associated with major side-effects of VIT.

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Venom immunotherapy (VIT) is an efficient and only viable option for prophylaxis in patients allergic to Hymenoptera venom (1). However, local and systemic side-effects of this treatment, especially during incremental phase of VIT, are not rare (2). Risk and pathogenic factors for those adverse reactions are so far poorly understood. VIT with honeybee venom induces side-effects more often than treatment with vespid venoms (3–6), which is further affected by different treatment protocols (7–9). The frequency of local and mild systemic reactions could be reduced by pretreatment with antihistamines (10), yet this is not obvious for the occurrence of severe anaphylactic reactions in patient treated with bee venom (11). Recently, the association between elevated serum tryptase level with or without mastocytosis and the severity of anaphylactic Hymenoptera sting reactions was shown (12, 13), but there is no evidence that basal serum tryptase level correlates to the frequency or severity of

side-effects to VIT. At the moment there is no reliable test, which can predict higher risk for adverse reactions during VIT. Flow cytometry quantification of basophil activation by measurement of CD63 expression (14) is a very sensitive and specific cellular *in vitro* method for identification of Hymenoptera venom allergy (13, 15, 16). As basophils play an important role in the pathogenesis of allergic anaphylactic reactions (17), we examined the hypothesis whether basophil responsiveness might be connected with side reactions to VIT. Therefore, the aim of our study was to evaluate, by measuring CD63 surface expression as a marker of basophil activation, whether there is any correlation between basophil *in vitro* sensitivity to allergen-specific stimulation and the occurrence of VIT-induced side-effects.

Materials and methods

Study subjects

Thirty-four patients undergoing VIT (median age of 41 years; age range: 17–70; 15 women) with a history of at least one systemic anaphylactic reaction of Mueller grades II–IV (18) after

Abbreviations: VIT, venom immunotherapy; BSB, basophil stimulation buffer; FcεRI, high affinity receptors for immunoglobulin E; fMLP, *N*-formyl-Met-Leu-Phe; *P*, probability; *r*, correlation coefficient.

Hymenoptera sting were included in the study. The sensitization was confirmed by skin prick testing and CAP-FEIA (Pharmacia, Uppsala, Sweden) measurement of specific immunoglobulin E (IgE). Additionally, measurement of serum tryptase was performed by CAP-FEIA (Pharmacia) just before the starting of VIT. Table 1 presents the characteristics of the study patients. The incremental phase of VIT was performed according to the modified rush protocol (1) reaching the maintenance dose (100 µg) of aqueous honeybee (*Apis mellifera*) or wasp (*Vespula* spp.) venom extracts (Hal Allergie, Duesseldorf, Germany; produced by Vespa Laboratories Inc., Spring Mills, PA, USA) within 2 days. Patients were pretreated with antihistamine loratadine, 10 mg daily. We also included a control group of subjects without a history of systemic reaction to Hymenoptera sting, nine women and one man with median age of 30 years and age range 24–46 years. All subjects were volunteers and gave their written consent after being fully informed about the purpose and nature of the study. The study was approved by the local ethics committee.

Basophil activation assay

Basophil activation test was performed by BD FastImmune (BD Biosciences, San Jose, CA, USA) in patients just before VIT or in control subjects. Briefly, whole blood (with heparin anticoagulant) was preincubated with basophil stimulation buffer (BSB) with interleukin (IL)-3 (19) containing a final concentration of 0.1 or 1 µg/ml of honeybee or wasp venom (Hal Allergie), 0.55 µg/ml of anti-high affinity receptors for immunoglobulin E (FcεRI) monoclonal antibody (mAb; Buehlmann Laboratories, Basel, Switzerland) or 2 µM *N*-formyl-Met-Leu-Phe (fMLP; Sigma, St Louis, MO, USA) at 37°C for 15 min. Chilling on ice stopped degranulation and thereafter fluorescein isothiocyanate (FITC)-conjugated anti-CD63 mAb, phycoerythrin (PE)-conjugated anti-CD123 mAb and PerCP-conjugated anti-HLA-DR mAb (BD Biosciences) were added and incubated for 20 min on ice. Finally whole blood probes were lysed, washed, fixed and analysed within 2 h on a FACSCalibur flow cytometer (BD Biosciences). Data were acquired with a threshold on FL2 set to eliminate most of CD123-negative cells, and

Table 1. Demographic data and diagnostic results in 34 patients undergoing VIT

Patient	Age	Sex	Culprit insect	Mueller grade	slgE (bee/wasp; kU/l)*	Tryptase (µg/l)†	Skin prick (bee/wasp)
1	54	M	Hornet	IV	4.75/3.97	4.92	Neg/Neg
2	44	F	Bee	III	12.5/0.65	3.11	100/100
3	44	M	Bee	IV	<0.35/<0.35	<1	Neg/Neg
4	23	M	Bee	III	6.37/<0.35	7.23	100/Neg
5	46	M	Wasp	III	2.32/0.85	4.65	ND/100
6	32	F	Bee	III	2.08/<0.35	2.85	100/Neg
7	51	M	Bee	III	3.63/0.93	2.06	100/ND
8	40	M	Bee	IV	<0.35/0.64	12.6	Neg/Neg
9	34	F	Bee	IV	3.59/0.4	4.26	100/Neg
10	40	F	Hornet	IV	2.18/2.36	2.26	100/100
11	17	M	ND	III	0.66/4.33	4.26	100/100
12	70	F	Bee	II	6.73/1.1	23.6	100/Neg
13	62	M	Wasp	IV	<0.35/<0.35	4.19	Neg/Neg
14	19	M	Bee	III	23.3/11.9	5.33	ND/ND
15	49	M	Wasp	IV	2.35/2.11	3.37	100/100
16	34	M	Bee	IV	0.61/2.57	8.76	100/100
17	62	F	Wasp	III	<0.35/2.40	3.02	100/100
18	41	F	Wasp	IV	0.39/0.9	5.57	ND/ND
19	35	M	Bee	III	2.25/0.45	14.1	10/Neg
20	39	F	ND	III	<0.35/0.68	2.02	ND/ND
21	57	F	Bee	IV	5.47/8.85	5.25	100/100
22	20	F	Bee	III	5.25/<0.35	3	100/Neg
23	49	M	Bee	III	3.27/<0.35	12.9	100/Neg
24	46	M	Bee	IV	10.2/1.02	4.79	Neg/Neg
25	43	M	Bee	IV	<0.35/<0.35	14.2	Neg/Neg‡
26	40	F	ND	II	<0.35/6.68	3.02	Neg/100
27	20	M	Hornet	IV	0.74/1.37	19.1	ND/ND
28	26	F	Wasp	IV	2.12/0.75	3.64	100/100
29	62	M	Wasp	IV	0.4/1.64	2.1	Neg/Neg
30	37	F	Hornet	IV	1.9/12.4	3.27	10/100
31	40	M	Wasp	IV	<0.35/1.37	39.2	100/100
32	19	F	Wasp	III	2.94/10.4	4.42	100/100
33	47	M	Wasp	IV	<0.35/<0.35	26.4	Neg/100
34	46	M	ND	IV	22.2/12	1.29	100/100

*slgE to bee and wasp venom were measured by CAP-FEIA.

†Baseline serum tryptase level was determined by CAP-FEIA just before starting of VIT (levels >10 µg/l were considered to be elevated).

‡Patient 25 had positive intradermal skin test result (bee venom, 0.1 µg/ml).

Skin prick test results indicate the allergen concentration (in µg/ml) eliciting a positive reaction.

ND, not determined; VIT, venom immunotherapy; slgE, specific immunoglobulin E; Neg, negative.

at least 600 CD123-positive cells per sample were acquired. Basophils were identified as low side scatter, CD123-positive and human leucocyte antigen (HLA)-DR-negative cells and the quantitative percentage determination of degranulated basophils was measured on FL1 (CD63).

Statistical analyses

For each patient we calculated the ratio between basophil CD63 expression after stimulation with allergen in concentration 0.1 and 1 µg/ml (0.1/1 ratio). The differences and strength of association between CD63 responses and side-effects to VIT or clinical grade of sting reactions was obtained by the Mann–Whitney test and Spearman rank order method. The incidence of side-effects to VIT or severe sting reactions in patients with normal *vs* elevated baseline serum tryptase levels was compared by using Fisher exact test. Probability values of $P < 0.05$ were accepted as significant.

Results

The data of basophil CD63 *in vitro* response in the control group are summarized in Table 2. Three of 10 control subjects had markedly increased CD63 response to honeybee venom and elevated levels of bee venom-specific IgE. Median percentage of basophil CD63 response to BSB (negative control), anti-FcεRI mAb or fMLP in control group was 2.8% (range: 0.7–5.8), 45.2% (range: 17.9–80.2) and 56.8% (range: 43–70.3), respectively. The data of the basophil CD63 response in patients undergoing VIT and detailed side-effects to VIT are summarized in Table 3 and Fig. 1. Median percentage CD63 response to stimulation buffer, anti-FcεRI mAb or fMLP in patient group was 2.7% (range: 1.8–5.8), 61.1% (range: 12.2–87.2) and 40.4% (range: 24.4–89.6), respectively. Eleven of 34 patients experienced systemic allergic reaction (eight were treated with honeybee and three with wasp venom, seven patients had Mueller grade I reaction, two patient grade II, two patients grade III and three of them had also large local reactions) and one patient large

local reaction (treated with wasp venom) to VIT. Eleven of them experienced side reactions during the incremental phase of VIT. In patients with side-effects, median percentage of activated basophils after stimulation with VIT-specific venom in concentration of 0.1 µg/ml was 99% (range: 17–195) of the value reached with stimulation with venom in concentration of 1 µg/ml. Side-effects occurred in all patients (eight of 12) with 0.1/1 ratio more than 92% and in four patients with ratios of 17%, 70%, 83% and 84%, respectively. In contrast, 22 patients with no side-effects, the median ratio was 25% (range: 2–92). These 0.1/1 ratios were significantly different between the patients without side-effects and patients with systemic reactions and/or local side reactions ($P < 0.0001$; Mann–Whitney test). Moreover, we also saw significant positive correlation ($r = 0.73$, $P < 0.0001$; Spearman rank order) for the severity of the side reactions with individual ratios of CD63 venom basophil response. There was no significant differences or correlation between the side-effects and the basophil CD63 response to FcεRI ($P = 0.17$; $r = -0.41$, $P = 0.1$; Mann–Whitney test; Spearman rank order) or fMLP ($P = 0.58$; $r = 0.08$, $P = 0.64$; Mann–Whitney test; Spearman rank order). Eight of 34 patients had elevated levels of serum tryptase (> 10 µg/l). In this group, five of eight patients (63%) had Mueller grade IV sting reactions and three of eight (38%) had side-effects to VIT in comparison with 14 of 26 (54%) with Mueller grade IV and nine of 26 (35%) with side-effects in a group with low tryptase levels ($P = 0.49$; $P = 0.72$; Fisher exact test). Nonsignificant results were observed when we compared clinical severity of the sting reactions and basophil CD63 *in vitro* responses.

Discussion

Our study was the first to evaluate basophil responsiveness in patients before receiving VIT for possible

Table 2. Results of sIgE detection, serum tryptase measurement and basophil *in vitro* venom response determined by the percentage of surface CD63-expressing CD123^{pos} HLA-DR^{neg} cells in 10 control subjects without a history of systemic reaction to Hymenoptera sting

Subject	sIgE (bee/wasp; kU/l)*	Tryptase (µg/l)*	0.1 µg/ml of bee venom (%)	1 µg/ml of bee venom (%)	0.1 µg/ml of wasp venom (%)	1 µg/ml of wasp venom (%)
1	<0.35/<0.35	6.3	4.9	6.9	4.9	5
2	<0.35/<0.35	5.7	5.6	2.5	3.8	5.6
3	<0.35/<0.35	3.6	3.7	3	4.1	2.5
4	0.35/<0.35	3.4	2.5	6.4	2.6	0.5
5	<0.35/<0.35	2.7	4.9	3.4	1.6	3
6†	0.35/<0.35	4.8	38.2	56.7	5.8	7.8
7†	1.5/<0.35	4.6	6.4	81	2.4	14.5
8	<0.35/<0.35	4.4	1.8	3.4	2.6	1.83
9	<0.35/<0.35	5.6	2.4	3.8	2.1	2.65
10†	0.47/<0.35	16.2	23.6	30.4	0.5	2.58

*sIgE and serum tryptase level were measured by using CAP-FEIA.

†Subjects with a history of large local reactions to Hymenoptera sting.
sIgE, specific immunoglobulin E.

Table 3. Basophil *in vitro* response determined by the percentage of surface CD63-expressing CD123^{pos} HLA-DR^{neg} cells in 34 patients before VIT and their side reactions during VIT

Patient	0.1 µg/ml of venom (A, %)*	1 µg/ml of venom (B, %)*	0.1 µg/ml of venom (%)†	1 µg/ml of venom (%)†	A/B (%)	VIT	Systemic side-effects‡	Large local reactions
1	13.4	88.8	1.8	31.9	15.0	Wasp	No	No
2	25.5	17.5	ND	2.7	145.3	Bee	Yes (III)	Yes§
3	36.5	75.0	ND	2.9	48.6	Bee	No	No
4	84.2	81.5	ND	1	103.4	Bee	Yes (I)	No
5	1.0	46.3	2.2	58.1	2.2	Wasp	No	No
6	9.5	55.9	ND	2.1	17.1	Bee	Yes (I)	No
7	7.9	56.0	ND	47	14.1	Bee	No	No
8	61.4	79.5	1.4	39.6	77.2	Wasp	No	No
9	86.5	44.4	6.1	57.6	194.6	Bee	Yes (I)	Yes
10	8.6	28.0	3.1	8.5	30.6	Wasp	No	No
11	50.6	86	3.4	3.6	58.9	Wasp	No	No
12	59.7	73.3	5.5	63.5	81.4	Bee	No	No
13	1.87	31.3	1.9	3.1	6	Wasp	No	No
14	8	76.8	12.6	88.4	10.4	Bee	No	No
15	7.7	47.5	37.1	91.8	16.2	Wasp	No	No
16	62.2	87.9	53.8	84.5	70.7	Bee	Yes (I)	Yes
17	65.3	70.9	4.5	9.9	92.1	Wasp	No	No
18	95.7	98.4	2.8	4.8	97.3	Wasp	No	Yes
19	61.3	61.7	0.9	38.1	99.4	Bee	Yes (II)	No
20	10.7	72.9	6.8	10.1	14.6	Wasp	No	No
21	8.7	16	6.2	15.2	54	Bee	No	No
22	89.3	70.4	2	4.3	126.8	Bee	Yes (III)	No
23	78.4	67	1.3	5.2	117	Bee	Yes (I)	No
24	45.3	81.7	33.9	76.5	55.4	Bee	No	No
25	12.7	39.9	ND	12.7	31.8	Bee	No	No
26	59.1	71.1	3.6	3	83.1	Wasp	Yes (I)	No
27	5.1	29.9	8.9	48.1	16.9	Wasp	No	No
28	26.6	87.5	34.3	94	30.3	Wasp	No	No
29	7	79.1	4.3	3.7	8.9	Wasp	No	No
30	6.4	66	18.3	41.6	9.6	Wasp	No	No
31	73.5	87.7	2.7	4.7	83.8	Wasp	Yes (I)	No
32	81.5	82.8	18.3	79.4	98.4	Wasp	Yes (II)	No
33	19.4	65.4	1.3	3	29.6	Wasp	No	No
34	15.6	79.2	6.3	86.2	19.6	Wasp	No	No

**In vitro* stimulation was performed with the same venom as VIT.

†*In vitro* stimulation was performed with family differing venom (bee or wasp) as VIT.

‡Systemic side-effects observed during incremental phase of VIT, except in patient 22. This patient had systemic anaphylactic reaction to maintenance dose after 4 years of treatment, which resulted in premature stoppage of VIT and restarting of treatment.

§Patient 3 repeatedly suffers from large local reactions.

VIT, venom immunotherapy; I, systemic reaction grade I (Mueller scale); III, systemic reaction grade III (Mueller scale); ND, not determined.

predicting of the adverse reactions. The focal point of our approach was not the measuring of basophil maximal response (13, 16), but the evaluation of basophil sensitivity (shift of the increasing dose-dependent activation) by recalculating CD63 response induced by two different log-allergen concentrations. The results showed that increased basophil sensitivity is associated with major side-effects during VIT and that monitoring of CD63 concentration-dependent venom response could be a relevant tool for identification of patients at higher risk for side-effects. Moreover, we also demonstrated a significant positive correlation between individual sensitivity ratio and clinical severity of side reactions.

Sensitivity was indicated as a percentage ratio between basophil CD63 response at 0.1 and 1 µg/ml of venom

dilutions. Those two allergen concentrations were selected because the majority of the reactors had peak basophil CD63 response at 1 µg/ml or at 0.1 µg/ml and because both concentrations do not produce nonspecific responses in control subjects. Basophil sensitivity significantly associated with side-effects was presented by higher or very close reactivity at 0.1 µg/ml than at 1 µg/ml of venom concentration. However, in two patients with side-effects of Mueller grade I we found a rather lower 0.1/1 ratio (17 and 70%), which suggests that other mechanisms could also be involved in mild adverse reactions. Therefore, it is important to stress that lower basophil sensitivity does not exclude the possibility of systemic reaction during VIT and that all patients should be carefully monitored during treatment. In agreement with

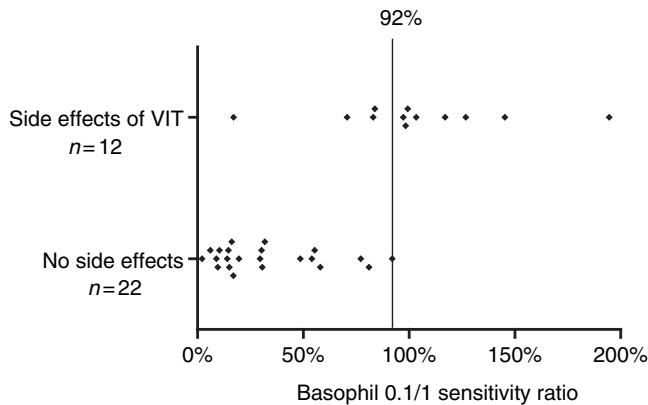


Figure 1. Basophil sensitivity determined as a percentage ratio between CD63 *in vitro* response at 0.1 and 1 µg/ml of venom dilutions in patients with ($n = 12$) and without ($n = 22$) side-effects to venom immunotherapy (VIT). Vertical line represents the highest 0.1/1 ratio in group without adverse reactions to VIT.

previous studies (13, 16), our preliminary observations showed that basophil CD63 concentration–response curves to bee or wasp venom are usually bell-shaped and rather highly individual from donor to donor (data not shown). Basophil sensitivity appears to be an independent intrinsic property connected with the intracellular signalling elements regardless of the cell surface density of the specific IgE (20, 21). We found no significant difference or correlation between side-effects and basophil response to direct cross-linking of the IgE receptor (anti-FcεRI mAb) or activation by G-protein-coupled transmembrane cell receptor (MLP). Both stimuli are frequently used as positive controls to confirm cell responsiveness. Our study was also the first to show correlation between clinical symptoms and basophil CD63 expression. Nevertheless, previous studies showed that surface up-regulation of CD63 is closely correlated with increased basophil histamine release (15, 19).

Significant adverse reaction at doses of <100 µg occurred in seven patients (47%) of 15 treated with honeybee venom; five patients had mild, one moderate and one severe systemic reactions. Similar incidence (40% of 15 patients) of systemic anaphylactic reactions during incremental phase of 2-day rush protocol with bee venom was reported by Rueff and Przybilla (7). However, the Reimers et al. (11) reported systemic reactions during bee venom ultrarush protocol in 26% of patients. In the subgroup treated with wasp venom only three patients (16%) of 19 experienced systemic adverse reactions, which is consistent with previous studies which clearly showed that bee venom induces side-effects more frequently than treatment with vespine venoms (3–6). Recent studies also suggest that side reactions tended to be more frequent during rush protocol with aqueous venom extracts than during conventional dose-increase schedule with depot venom preparations (8, 9). These clinical

observations suggest that the side-effects could also be associated by the different dynamics of the *in vivo* venom appearance during dose-increase phase of VIT. Hence, we can speculate, as it was shown that basophils partially degranulate during VIT (22) that threshold for adequate basophil activation necessary for adverse reactions is reached more frequently in patients with very sensitive basophils than in patients with relatively low-sensitive basophils.

In agreement with previous studies (13, 15, 16), we demonstrated that CD63 activation test is a valuable diagnostic tool in Hymenoptera venom allergy. All of our patients were positive with the cut-off of 15% of CD63-positive basophils (16). Three control subjects also had positive CD63 response (only bee venom); however they had elevated levels of bee venom -specific IgE and histories of large local reactions to honeybee stings. Interestingly, three patients who experienced very severe sting reactions (Mueller grade IV) had no detectable specific IgE and negative skin prick test; however all of them had positive CD63 response to culprit insect venom. In those complex cases with inconclusive results of specific IgE antibodies and skin test response, CD63 activation test could be particularly useful. Double sensitization to both honeybee and wasp venom is another problem in diagnosis of patients allergic to Hymenoptera venom, posing difficulties in the selection of venom for treatment. Interestingly, three of 10 patients with elevated specific IgE and positive skin test for both venoms had positive CD63 response only for a single venom. Similar findings were recently observed by Sturm et al. (13). We could only speculate that the basophil stimulation test does not detect clinically irrelevant IgE against carbohydrate epitopes, which are a common cause of double sensitivity in IgE assays (23). However, inhibition experiments (24) are needed to confirm this hypothesis.

We could not demonstrate that serum tryptase was a relevant prognostic marker for the severity of anaphylactic sting reactions, as has been suggested earlier (12, 13). Nor could we demonstrate a correlation between the CD63 responses and severity of clinical symptoms after a sting, which is in agreement with previous report (13). In addition, we could not show that patients with elevated tryptase were at increased risk of side-effects during VIT. Therefore, determination of serum tryptase is not a useful predictor of individual risks of side-effects.

In conclusion, our study established a possible model for the predicting of individual risk of adverse reactions during VIT. The results suggest that specific basophil sensitivity is significantly associated with major side-effects and their clinical severity during VIT.

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