

Original article

The CD63 basophil activation test in Hymenoptera venom allergy: a prospective study

Background: The basophil activation test (BAT), which relies on flow cytometric quantitation of the allergen-induced up-regulation of the granule-associated marker CD63 in peripheral blood basophils, has been suggested to be a useful approach in detecting responsiveness to allergens. The purpose of this study was to establish the usefulness of the BAT with regard to the clinical history and current diagnostic tools in Hymenoptera venom allergy using a prospective study design.

Methods: Fifty-seven consecutive patients allergic to Hymenoptera venom as defined by a systemic reaction after an insect sting, and 30 age- and sex-matched control subjects with a negative history were included. The degree and nature of sensitization was confirmed by skin testing, specific immunoglobulin E (IgE), serum tryptase levels and BAT. In the nonallergic control group only analysis of specific IgE and BAT were performed. Correlation of BAT, skin test and specific IgE, respectively, with the clinical history in the allergic group was termed as sensitivity and in the control group as specificity.

Results: Twenty one of 23 (91.3%) bee venom allergic patients and 29 of 34 (85.3%) patients allergic to wasp and hornet venom tested positive in BAT. The overall sensitivity of BAT, specific IgE and skin tests were 87.7, 91.2 and 93.0%, respectively. The overall specificities were 86.7% for BAT and 66.7% for specific IgE. No correlation between the severity of clinical symptoms and the magnitude of basophil activation was observed.

Conclusion: The BAT seems to be an appropriate method to identify patients allergic to bee or wasp venom with a comparable sensitivity to standard diagnostic regimens. The higher specificity of BAT as compared with specific IgE makes this test a useful tool in the diagnosis of Hymenoptera venom allergy.

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Up to 5% of the general population are reported to have experienced systemic hypersensitivity reactions to Hymenoptera stings (1). Hymenoptera venom immunotherapy is a well-established treatment in these patients and offers full protection from further generalized reactions in 83–95% of persons with a history of anaphylactic reactions after an insect sting (2–4). Current diagnostic regimens of insect venom allergy, based on allergen-specific immunoglobulin E (IgE) serum antibodies [radioallergosorbent test (RAST), CAP-FEIA] and skin testing (skin prick test, intradermal test), may be sufficient in most cases, but there is still concern about their sensitivity and specificity. The sensitivity of skin testing was found to range from 63 to 100%, depending on the protocols and venoms used, and with specificity ranging from 80 to 97% (5–8). By comparison, the sensitivity of

RAST is estimated as 70 to 76%, and specificity as 60 to 94% (7, 9–11). As a major obstacle of these tests, positive responses in skin testing and high levels of specific IgE antibodies are not predictive for the occurrence and the severity of systemic reactions after a field sting, or a sting challenge test (1, 12, 13), or unwanted episodes during immunotherapy (14). Recently, the basophil activation test (BAT) has been referred to as a highly sensitive and specific method to detect Hymenoptera venom allergy, with reported sensitivity and specificity being as high as 100% (15). These promising results, however, need be confirmed with a higher number of patients in a prospective study. This novel assay is based on the fact that in resting granulocytes CD63 is located within the granule membranes and absent from the cell surface, but during exocytosis, when the granule membrane fuses with the cell membrane, it is translocated to the cell surface. In basophils, a close correlation between CD63 expression

Abbreviation: BAT, basophil activation test.

and histamine release has been demonstrated (16). The levels of CD63 expression can be readily quantified by flow cytometry using specific antibodies directed against CD63 and conjugated to fluorescent dyes. Therefore, BAT is a simple and fast *in vitro* assay and has been suggested to be a useful procedure in the diagnosis of food (17), pollen (18), dust mite (19), drug (20, 21), latex (22), and also Hymenoptera venom allergy (15). The aim of the current study was to determine the clinical usefulness of BAT in the diagnosis of venom allergy using a prospective study design and to compare those results with currently employed regimens, i.e. skin testing and IgE detection. In order to establish an optimal protocol regarding sensitivity and specificity of BAT a wide range of allergen concentrations was tested in each patient. Furthermore, the hypothesis was scrutinized whether BAT might be suitable for predicting the individual risk for a severe anaphylactic reaction.

Material and methods

Selection of patients

Fifty-seven patients, who had been admitted to our outpatient clinic because of systemic allergic reactions with a clinical grade I or higher after a Hymenoptera sting, were consecutively included in the study on the basis of clinical history. Patients were included before any test procedure (i.e. skin testing, IgE detection and BAT) and were not selected in any way. Twenty-three patients reported a bee sting, 28 a wasp sting and six a European hornet sting. As wasp and European hornet belong to the family of Vespidae and their venoms contain the same major antigens (23), we did not differentiate between these genera. Thirty age- and sex-matched subjects without a history of systemic allergic reactions after a sting served as controls. None of the patients/control subjects were on systemic medication at the time of testing. Time point of testing was at least 2 weeks after the initial sting reaction, the median sting-to-test interval was 10 weeks (range 2–312 weeks).

Skin tests

The nature of sensitisation was confirmed by standardized end-point titration skin prick (10, 100, and 300 µg/ml solution) and intradermal tests (0.05 ml of 0.1 and 1 µg/ml solution) using purified bee and wasp venom extracts (ALK-Abello, Frederiksberg, Denmark). Prick tests and intradermal tests were considered to be positive if a wheal of ≥3 and 5 mm in diameter with erythema was observed, respectively. In the control group no skin tests were performed for ethical reasons.

Specific IgE determination

Measurement of specific IgE antibody levels in the patients' serum was performed by CAP-FEIA (Pharmacia, Uppsala, Sweden), according to the manufacturer's instructions.

Tryptase determination

Measurement of serum tryptase was performed by UniCAP Tryptase (Pharmacia) according to the manufacturer's instructions

at least 2 weeks after the initial sting reaction. Baseline serum tryptase levels were determined to exclude mastocytosis as a reason of nonspecific reactions to the sting. Levels >9.8 µg/l (90th percentile of the general population) were considered to be elevated.

Basophil activation test

Basophil CD63 up-regulation in response to stimulants was determined as previously described for eosinophils (24). Citrated whole blood was layered over Histopaque 1077 (Sigma, Vienna, Austria) and centrifuged at 300 *g* for 20 min. The resulting layer of mononuclear cells comprising basophils, lymphocytes and monocytes was collected and washed twice in assay buffer without $\text{Ca}^{++}/\text{Mg}^{++}$ (phosphate-buffered saline supplemented with 0.1% bovine serum albumin, 10 mM HEPES, and 10 mM glucose, pH 7.4) and stained with anti-CDw123 phycoerythrin-conjugated antibody (Becton-Dickinson, Vienna, Austria) and anti-HLA-DR quantum red-conjugated antibody (Sigma). After a final wash cells were resuspended in assay buffer with $\text{Ca}^{++}/\text{Mg}^{++}$ at 2×10^6 cells/ml and incubated with cytochalasin B (5 µg/ml; Sigma) and anti-CD63 fluorescein isothiocyanate-conjugated antibody (Autogen Bioclear, Calne, UK) for 5 min at 37°C. Twenty-five microlitre aliquots of cells were then added to 25 µl of the respective agonist or buffer in 1.1 ml polypropylene microtubes (Bioquote Ltd, York, UK). Cells mixed with assay buffer were used as negative control to determine expression of CD63 in the absence of a stimulant, while cells mixed with C5a (25 nM; Sigma) or anti-IgE antibody (1 : 100–1 : 1 000 000 dilution; Sigma) served as positive controls to confirm cell responsiveness. Cell responsiveness to allergens was determined using six serial dilutions of bee and wasp venom (10, 1, 0.1, 0.01, 0.001 and 0.0001 µg/ml; ALK-Abello). The microtubes (including positive and negative controls) were placed in a 37°C water bath for 30 min, a period which we found to give optimal responses in CD63 up-regulation in preliminary experiments. The samples were then transferred to an ice water bath for 5 min to terminate the reaction and washed in ice-cold assay buffer. Finally, cells were resuspended in 100 µl of ice-cold fixative, prepared from Cellfix by 1 : 10 dilution in water and 1 : 4 dilution in FACSFlow (both from Becton-Dickinson, Vienna, Austria). Cell samples were analysed by three-colour flow cytometry (FACScalibur; Becton-Dickinson, Mountain View, CA). Basophils were identified as a single population of cells that stained positive for CDw123 (FL-2 fluorescence channel) and negative for HLA-DR (FL-3) (Fig. 1A). Up-regulation of CD63 in basophils was indicated by an increase in fluorescence in the FL-1 channel. Acquisition was terminated after 150 basophil target events. Responses were quantified as percentage of CD63 expressing basophils found in a high-FL-1 region, which had been adjusted to contain 10% of basophils and defined as 'activated basophils', in an unstimulated sample (negative control; Fig. 1B). An approximately 2.5-fold increase in the number of activated basophils (>25%) as compared with the negative control (10%) at any of the test concentrations of the allergen (0.0001–1 µg/ml) was considered to be a positive response (Fig. 1C).

Data analysis

The sensitivity was defined as the percentage of positive tests obtained for the Hymenoptera venom allergic patients according to their clinical history. Specificity was defined as the percentage of negative tests in the control group. Sensitivities and specificities for each venom and the overall sensitivities and specificities for each test were calculated, and data were compared with chi-square test. A possible correlation of the magnitude of the CD63 response to the

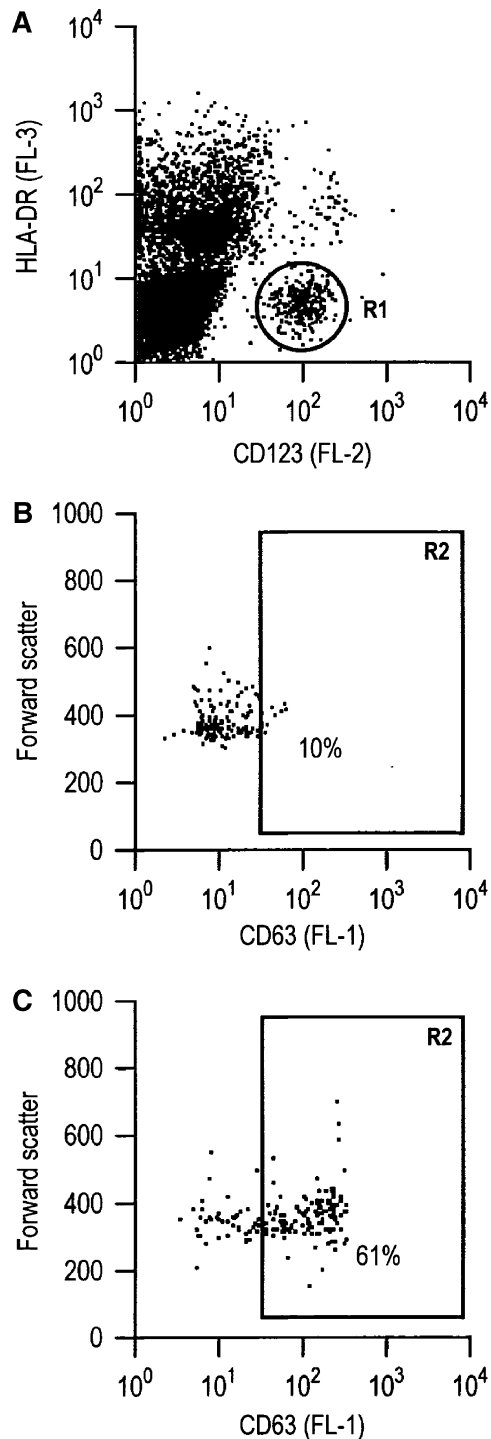


Figure 1. Quantitation of allergen-induced CD63 up-regulation in basophils. Basophils were identified as CDw123^{pos}/HLA-DR^{neg} cells (R1; A). Up-regulation of CD63 in basophils was quantified as percentage of CD63 expressing basophils found in a high-FL-1 region (R2), which had been adjusted to contain 10% of basophils and defined as ‘activated basophils’, in an unstimulated sample (negative control; B). Incubation of cells with allergen resulted in an increase of CD63-positive cells in R2 (61%), as shown for a patient allergic to wasp venom (C).

allergen and the severity of clinical symptoms was determined using ANOVA on ranks (Kruskal–Wallis *H*-test). The incidence of severe clinical symptoms in patients with normal vs elevated serum tryptase levels was compared using Fisher Exact test. The optimal threshold to differentiate between negative and positive results was determined by receiver-operated curve (ROC) analysis. Positive predictive values were calculated as numbers of true positives divided by all positives, and negative predictive values as true negatives divided by all negatives.

Results

Of the 57 patients included in this study, five experienced a grade I reaction [classification according to Ring and Messmer (32)], 35 a grade II reaction, and 17 a grade III reaction. The BAT, skin testing and specific IgE detection were performed for both allergens, bee and wasp venoms, irrespective of the clinically relevant species. Similarly, BAT and allergen specific IgE detection were performed for both allergens in the 30 control subjects. Evaluation of BAT results of all patients and control subjects with ROC curves using different threshold values (20–30% activated basophils) showed that a threshold of 25% gave optimal sensitivity and specificity (area under the curve of 0.917, standard deviation 0.0281), as based on clinical history. The concentration–response curves to allergens were bell-shaped in many cases, with the maximum response being observed at intermediate allergen concentrations, and the number of activated basophils decreased at supra-maximal concentrations. Figure 2 depicts the distribution of the maximal responses with regard to allergen test concentration showing that in a considerable

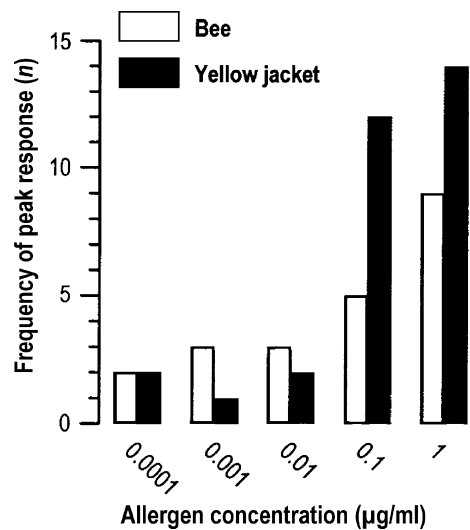


Figure 2. Peak responses to bee and wasp venom in basophil activation test with regard to allergen test concentration. The bars show the number of patients, who exhibited their maximal CD63 response at the respective allergen dilution.

Table 1. Results of immunoglobulin E (IgE) detection, skin testing and basophil activation test

Patient	Culprit insect	Clinical grade	Tryptase (µg/l)	IgE bee (class)	IgE wasp (class)	Total IgE (kU/l)	ST positive for bee at (µg/ml)	ST positive for wasp at (µg/ml)	Anti IgE %max	C5a %max	Bee %max	Wasp %max
1	Wasp	2	6.6	3	4	281.0	1.0	1.0	38.6	64.1	41.5	52.1
2	Wasp	3	3.5	0	2	49.7	Neg.	300	84.7	79.6	14.1	17.9
3	Wasp	2	4.5	0	2	72.5	Neg.	300	74.4	73.9	39.6	82.8
4	Bee	2	5.5	3	0	43.9	100	Neg.	56.1	65.1	57.0	22.5
5	Bee	2	7.1	2	0	232.0	1.0	1.0	27.7	60.9	28.1	23.2
6	Hornet	2	5.0	0	2	21.2	Neg.	1.0	32.0	48.5	15.2	29.7
7	Wasp	3	28.3	0	0	2.8	Neg.	Neg.	67.8	76.9	23.6	25.9
8	Bee	2	27.7	2	1	331.0	1.0	1.0	68.4	58.6	40.7	12.2
9	Bee	2	5.0	2	1	40.1	300	300	47.4	74.5	28.6	21.4
10	Hornet	3	4.2	2	2	69.8	Neg.	300	70.5	84.0	20.2	81.3
11	Bee	3	101.0	4	3	125.0	300	300	36.4	50.6	38.2	32.2
12	Bee	2	3.8	6	2	556.0	300	Neg.	22.7	6.5	26.9	30.7
13	Wasp	3	2.2	2	3	132.0	Neg.	300	34.8	74.3	18.8	29.5
14	Bee	2	1.4	3	2	52.3	100	100	26.1	9.4	46.6	29.1
15	Wasp	2	6.1	0	1	42.7	Neg.	300	20.4	75.2	19.5	17.6
16	Wasp	3	4.5	4	3	343.0	300	300	24.3	17.1	33.9	48.5
17	Wasp	1	4.6	0	2	40.7	100	Neg.	37.7	59.3	16.8	59.4
18	Wasp	1	1.0	2	2	147.0	100	100	66.3	71.9	54.6	71.1
19	Wasp	2	6.6	0	3	42.3	Neg.	100	42.6	46.5	16.3	54.8
20	Hornet	2	3.1	2	2	17.7	100	100	63.6	85.5	24.3	22.8
21	Bee	2	2.0	0	0	0.0	1.0	Neg.	79.3	60.1	33.1	14.9
22	Wasp	2	13.3	2	2	38.0	300	1.0	72.0	86.8	80.7	71.1
23	Wasp	1	4.4	2	2	99.0	1.0	1.0	54.5	77.0	26.9	70.4
24	Bee	2	21.5	2	1	34.0	300	Neg.	40.1	83.3	52.8	35.7
25	Wasp	3	28.1	0	0	32.5	Neg.	1.0	17.9	61.3	21.3	15.1
26	Bee	2	8.7	2	1	45.5	300	300	22.4	22.3	67.7	62.3
27	Hornet	2	6.3	1	2	50.5	Neg.	300	66.5	95.7	30.0	60.0
28	Hornet	2	2.0	1	2	172.0	Neg.	100	69.5	50.0	13.9	34.7
29	Wasp	3	25.8	0	1	12.4	Neg.	300	15.2	6.2	21.4	31.5
30	Wasp	2	4.2	2	3	86.2	300	0.1	21.3	52.1	6.2	25.3
31	Bee	3	5.8	2	2	40.5	300	300	80.7	66.2	72.7	35.4
32	Wasp	3	8.3	2	3	158.0	300	1.0	42.9	95.7	28.9	55.7
33	Wasp	2	7.9	0	3	17.1	Neg.	300	52.4	67.9	29.4	55.8
34	Wasp	2	5.2	3	2	78.0	100	100	59.1	76.8	27.5	26.0
35	Bee	2	1.7	6	3	1326.0	300	300	41.2	65.1	60.5	18.3
36	Bee	3	10.5	2	2	45.7	300	300	55.2	40.0	55.6	52.1
37	Bee	2	3.2	3	1	24.5	300	1.0	59.4	63.5	49.7	28.6
38	Bee	2	2.4	5	3	440.0	300	Neg.	19.6	78.5	29.7	26.8
39	Bee	2	5.1	3	3	150.0	100	100	63.2	54.8	46.7	21.5
40	Wasp	2	7.7	0	1	11.7	Neg.	300	34.8	40.6	9.4	38.0
41	Wasp	2	5.3	1	5	151.0	Neg.	100	69.4	54.3	20.9	40.7
42	Hornet	1	5.0	3	2	24.7	0.1	1.0	59.8	43.3	67.7	63.8
43	Bee	3	4.3	4	3	137.0	100	Neg.	80.7	64.6	72.1	37.8
44	Wasp	2	3.6	3	2	53.5	100	100	91.6	84.7	60.6	86.4
45	Bee	1	7.6	3	3	212.0	Neg.	Neg.	43.3	90.4	32.1	38.0
46	Wasp	3	6.9	5	4	223.0	100	100	20.3	91.5	33.3	28.7
47	Wasp	3	2.3	0	2	169.0	Neg.	100	25.7	35.2	17.6	56.0
48	Bee	2	2.2	3	2	71.6	300	300	26.2	78.5	56.2	19.9
49	Wasp	3	6.2	0	0	47.2	Neg.	Neg.	57.9	79.2	41.5	57.1
50	Bee	2	4.3	3	1	68.9	100	0.1	43.8	53.7	45.1	26.6
51	Bee	2	5.5	3	2	140.0	100	100	38.6	65.2	16.3	9.8
52	Bee	2	4.4	3	0	61.0	1.0	Neg.	55.5	44.3	10.8	11.6
53	Wasp	2	10.7	0	0	13.1	300	300	69.2	48.0	16.4	21.4
54	Bee	2	5.9	5	2	378.0	100	0.1	71.3	75.0	87.4	22.1
55	Wasp	3	7.0	0	2	17.2	Neg.	100	55.2	58.3	23.6	51.6
56	Wasp	2	5.4	1	3	41.2	Neg.	300	78.5	57.3	18.7	91.1
57	Wasp	3	12.1	3	3	312.0	100	100	21.6	71.5	16.1	39.7

Clinical grade: classification according to Ring and Messmer (32).

Specific IgE classes: 0, <0.35 kU/l; 1, 0.35–0.70 kU/l; 2, 0.70–3.5 kU/l; 3, 3.5–17.5 kU/l; 4, 17.5–50 kU/l; 5, 50–100 kU/l; 6, >100 kU/l.

ST, skin test; 100–300 µg/ml, prick test; 0.1–1.0 µg/ml, intradermal test; %max, maximal percentage of activated basophils; Neg., negative.

number of cases the peak response occurred at very low allergen concentrations.

The data of the individual test subjects are summarized in Table 1. According to this protocol 21 of 23 patients allergic to bee venom and 29 of 34 allergic to wasp venom tested positive to the respective allergen in BAT. Elevated levels of specific IgE were detected in 22 of 23 patients allergic to bee venom and 30 of 34 allergic to vespid venom. Positive results in the skin tests were observed in 22 of 23 patients allergic to bee venom and in 31 of 34 patients allergic to wasp venom. Eleven of 53 positive skin tests gave positive responses only at 1.0 µg/ml. Three of the 30 control patients showed positive results for bee venom, and five for wasp venom in BAT. Two of them had experienced marked local reactions after a sting; in the remaining six patients BAT presumably revealed a clinically irrelevant sensitization. Nine of the 30 control subjects had detectable levels of IgE antibodies for bee venom, and 11 for wasp venom up to class 4. All eight control subjects, who tested positive in BAT, also had elevated specific IgE. None of the controls, who tested positive, reported systemic allergic reactions in the past.

Positive results for both bee and wasp venom were obtained by the measurement of specific IgE in 39 patients, skin testing in 29 cases and BAT in 19 of 57 patients. Of nine cases showing double sensitization with nearly equal levels of specific IgE for bee and wasp venom, and positive results in the skin tests for both venoms, BAT was positive only for a single venom in four cases. Of the five patients with no detectable specific IgE in the insect-allergic group, three were positive in BAT, while the four skin test negatives had positive results in BAT. Of those seven patients, who were unresponsive in BAT, five patients were positive both for specific IgE and in skin test. Of the remaining two patients with negative BAT and no detectable specific IgE one patient had an elevated serum tryptase level and a positive skin test at 1.0 µg/ml wasp venom, the second patient had positive skin test results at 0.1 µg/ml for both bee and wasp venom.

A comparison of the sensitivities and specificities of the different tests are shown in Table 2. While the sensitivities of BAT, specific IgE and skin testing were similar, the specificity of BAT was significantly higher than that of specific IgE ($P = 0.017$, Chi-square test). Positive predictive values for BAT are 86.2 and 72.2% for specific IgE and, derived from published literature, ranging between 50.0 and 96.9% for skin testing (5, 7). Negative predictive values are 88.1% for BAT and 88.9% for IgE, and for skin testing between 87.0 and 90.5% (5, 7). All patients who were negative in the skin tests had positive results in BAT and conversely all patients negative in BAT had positive responses in the skin tests. Thus, the combination of BAT and skin testing achieved a sensitivity of 100%.

Finally, we tested the hypothesis, that BAT may be suitable to predict the severity of the anaphylactic

Table 2. Sensitivity and specificity (%) of basophil activation test (BAT), specific immunoglobulin E (IgE), and skin testing in bee and wasp venom allergy

	BAT		Specific IgE		Skin tests	
	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
Bee	91.3	90.0	95.7	70.0	95.7	nd
Wasp	85.3	83.3	88.2	63.3	91.2	nd
Overall	87.7	86.7	91.2	66.7	93.0	nd

Twenty-three patients allergic to bee, 34 to wasp venom, and 30 control subjects were tested with the indicated allergens in the three tests. Sensitivities and specificities were calculated with respect to clinical history. nd, not determined.

reaction because of a sting. Therefore, a possible correlation between clinical grading of the reported reaction and the magnitude of CD63 up-regulation in response to Hymenoptera venom was investigated. We found no significant differences in the degree of basophil activation in BAT between the groups of patients with clinical grade I, II and III ($P = 0.095$, ANOVA on ranks). Moreover, there was no correlation of the clinical grades with the individual sensitivity to the venom in BAT, as inferred from the allergen test concentration at which the peak effect was observed ($P = 0.45$, ANOVA on ranks). Of the 57 patients 10 had elevated levels of serum tryptase (> 9.8 µg/l). In this group significantly ($P = 0.022$, Fisher Exact test) more patients (60.0%) experienced clinically grade III reactions than in the group with low tryptase levels (23.4%). All patients with high tryptase levels were found to be positive in at least one of the three test methods.

Discussion

In the present study we demonstrate that BAT seems to be an appropriate and useful procedure in diagnosing Hymenoptera venom allergy. We found that the sensitivities of BAT, skin testing and determination of specific IgE antibodies by CAP-FEIA were comparable, but in terms of specificity BAT was superior to specific IgE detection. In addition, our data point to methodologically relevant issues which might help to avoid possible pitfalls of this assay. The sensitivities of BAT, specific IgE and skin tests were comparable (87.7, 91.2 and 93.0%, respectively), but specificity of BAT was superior to specific IgE detection (86.7% vs 66.7%). The sensitivity of skin testing might have even been overestimated in the current study, as 11 of 53 positive skin tests gave positive responses only at 1.0 µg/ml of the allergens, a dose which has been reported to cause nonspecific reactions rather frequently (7, 25). If these patients were recorded as negative, the sensitivity of skin testing would be as low as 73.7%. On the basis of these data BAT seems to be at least as reliable as skin

testing in the diagnosis of Hymenoptera venom allergy. Moreover, BAT combined with skin tests had a sensitivity of 100%, as all patients who were negative in the skin tests had positive results in BAT and, conversely, all patients negative in BAT had positive responses in the skin tests. Therefore, BAT may be particularly useful in cases, where skin testing contrasts clinical history and, because of their low specificity, specific serum IgE levels cannot be relied on in this respect.

Cross-reactivity of bee and wasp venoms in skin testing and specific IgE is a frequent clinical problem mimicking double-sensitization, thus posing difficulties on which venom the patient should be made tolerant to. Interestingly, four of nine patients with the same CAP-classes for bee and wasp and with positive results at the same dilutions in the skin tests, were positive only for a single venom in BAT. Further studies including sting challenge are needed to confirm whether BAT is suitable to distinguish between clinically relevant sensitization and cross-reactivity between similar venom components.

We could not demonstrate a positive correlation of the magnitude of BAT responses and severity of clinical symptoms after a sting. Similar findings have been previously reported for skin testing and specific IgE levels in serum (12, 13). Therefore, it is apparently impossible to predict the individual risk of severe sting reactions with BAT. In contrast, we found that serum tryptase level might be a relevant prognostic marker for the severity of anaphylactic reactions to Hymenoptera venom in allergic patients, as 60.0% of patients with elevated tryptase levels reported grade III reactions caused by a previous sting, while only 23.4% of patients with low tryptase levels had experienced grade III reactions. Therefore, elevated serum tryptase levels in sensitized individuals could indicate a higher risk of severe sting reactions, as has been suggested earlier (26, 27). These patients tested positive in, at least, one of the three test methods, but the degree of sensitization seemed to be lower as compared with patients having normal serum tryptase levels. Therefore, determination of serum tryptase levels in patients with no or low sensitization, but with a history of severe sting reactions, might help to estimate their risk more accurately.

In the current approach basophils were identified by double-staining of mixed leukocytes with antibodies directed against HLA-DR and CDw123 (α -chain of the IL-3 receptor). This resulted in a single population of HLA-DR-negative/CDw123-positive cells, which had previously been shown to be basophils (28). Importantly, we observed that expression levels of these markers were largely invariable in all subjects tested (29). In agreement with this, Siegmund et al. (30) found that CD123 expression is similar in allergic and nonallergic patients, although they found a slight increase of HLA-DR expression in allergic patients.

Therefore, basophils could be identified by flow cytometry in all cases. In contrast, some authors (15) and commercially available tests utilize anti-IgE antibodies in order to identify basophils, and the assays might fail in subjects with low levels of IgE on their basophils. Low levels of IgE, however, do not preclude functional responsiveness to a particular allergen. Recently, basophil up-regulation of CD203c expression was described as a highly sensitive marker of allergen responsiveness. CD203c up-regulation in Hymenoptera venom allergic patients correlated well with the CD63 expression and histamine release and, thus, might be a useful alternative approach (31).

Another issue addressed by the current study was to establish the optimal test concentrations of the allergens in BAT. Out of the six allergen dilutions (10, 1, 0.1, 0.01, 0.001 and 0.0001 $\mu\text{g/ml}$) the highest concentration of 10 $\mu\text{g/ml}$ caused basophil activation above threshold in a high number of control subjects suggesting a nonspecific effect and was therefore excluded from further analysis. These false-positive responses might have been due to enzymatic activity of the venoms or nonspecific allergen effects. Such responses were not observed at allergen concentrations of 1 $\mu\text{g/ml}$ or below. In allergic patients basophil responses exhibited a correlation with allergen test concentration, but after the peak response had been reached, the number of activated basophils decreased at higher concentrations in many cases. Eventually even sub-threshold responses were observed at higher allergen concentrations in some patients, who clearly gave positive results at low allergen concentrations. The peak response was usually observed at the concentrations of 1 and 0.1 $\mu\text{g/ml}$, but in many cases the maximal response occurred even at very low allergen concentrations (Fig. 2). From these data we inferred that a wide range of allergen dilutions was mandatory in order to circumvent the possibility of false-negative results because of blunted responses at supra-maximal test concentrations. Therefore, it seems recommendable for routine use to test with three dilutions (1, 0.1, 0.01 $\mu\text{g/ml}$).

On long terms two scenarios are conceivable: BAT could be useful, if additional information to specific IgE measurement and skin testing is needed. On the contrary, simplified commercial kits for BAT using automated flow cytometers could be used as first-line diagnostic procedure given that the specificity of BAT is considerably higher than that of specific IgE.

In conclusion, BAT is a useful diagnostic regimen in Hymenoptera allergy, with a sensitivity comparable with standard diagnostic tools, skin testing and specific serum IgE, while the specificity of BAT is exceeding that of specific serum IgE. Furthermore, our data suggest that BAT may be particularly useful in complex cases, such as suspected double-sensitization or inconclusive results with skin testing and specific IgE detection in patients having experienced severe sting reactions. These data on

sensitivity and specificity will have to be confirmed by long term follow-up of patients with respect to future reactions to field sting, in order to establish the predictive value of BAT.

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