

Use of CD63 expression as marker of in vitro basophil activation in identifying the culprit in insect venom allergy

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ABSTRACT

Background: The diagnosis of insect venom allergy and the indication for specific immunotherapy is based on history, skin tests and demonstration of hymenoptera venom specific IgE-antibodies. In cases with contradictory test results additional cellular tests are recommended.

Objective: We evaluated the usefulness of a newly introduced test based on basophil CD63 expression as marker of activation in comparison with the basophil histamine release test (BHR) and the cellular antigen stimulation test (CAST) measuring leukotriene release.

Methods: In 14 patients (10 males, 4 females; age: 12 to 67 years, mean: 42.5 ± 15.1 years) with systemic reactions to hymenoptera stings in their history skin tests and determination of specific IgE-antibodies (CAP-RAST-FEIA) had shown inconsistent results: No demonstration of specific IgE-antibodies (n=4), one sting by an unknown insect together with positive skin test and/or positive RAST to both bee and wasp venom (n=4), several stings of partly unknown insects with positive skin test and/or demonstrable specific IgE-antibodies to more than one insect venom (n=4), uncertain history and divergent results in skin test and/or RAST (n=2). BHR, CAST and basophil activation test (BAT) were done according to the manufacturers with negative and positive controls and different concentrations of bee and wasp venom. The BAT is based on double staining with anti-IgE antibodies and anti-CD63 and subsequent determination of the percentage of activated basophils by flow cytometry.

Results: BAT and skin test were concordant in 42.9%, BAT and RAST in 57.1%. Concordance of all three cellular tests was seen in 57.1%, of BAT and BHR in 69.1%, of BAT and CAST in 78.6% and of BHR and CAST in 64.3%. In 6 cases where the three cellular tests (BHR, CAST, BAT) were not in accordance the addition of BAT led to a more reliable diagnostic result concerning the relevant insect in 3 cases and added no further information in 3 cases. BAT in controls always was negative. Correlation between CAST and BAT was higher than between CAST and BHR.

Conclusions: In difficult cases of hymenoptera allergy, where history, skin tests and determination of specific antibodies do not allow a clear decision regarding the relevant insect species for immunotherapy, the additional performance of cellular tests (CAST and BAT) may be helpful.

Key words: basophils, flow cytometry, histamine, hymenoptera venom allergy, leukotrienes, specific immunotherapy.

Introduction

Life threatening, anaphylactic reactions to hymenoptera stings occur in 0.8-5% of the general population [1]. Treatment of this IgE-mediated allergy with specific

immunotherapy is highly effective: about 80-100% of those who formerly had reacted systemically do not so when re-stung after treatment has been initiated [2]. The diagnosis of insect venom allergy and the indication for specific immunotherapy is based on history, skin testing

and demonstration of hymenoptera venom-specific IgE antibodies. Information regarding the insect species is often insufficient, because many people cannot distinguish with certainty between bees and wasps. In cases with contradictory test results additional cellular tests like histamine release or cellular antigen stimulation test are recommended. In such cases, we have assessed the additional usefulness of the basophil activation test, based on CD63 expression on basophils as marker of activation.

Material and methods

Patients

Fourteen patients (10 men, 4 women, age 12 to 67 years, 42.5 ± 15.1) and five controls (2 men, 3 women, age 29 to 60 years, 42.2 ± 14.7) were selected for the study on the basis of discrepant results in allergy diagnosis.

All patients had a history of systemic reactions to hymenoptera stings. Threshold intracutaneous testing with honeybee or yellow jacket venom (Venomil; Bencard, Munich, Germany) was done on the ventral aspect of the forearm with incremental concentrations of 0.0001, 0.001, 0.01 and 0.1 $\mu\text{g/ml}$. Venom-specific serum IgE-antibodies were determined by a fluorescence enzyme immunoassay (Pharmacia CAP, RAST FEIA, Uppsala, Sweden). In patients, history, skin test and determination of specific IgE-antibodies had shown inconsistent results:

- 1) Uncertain history and no demonstration of specific IgE-antibodies (n=4)
- 2) Uncertain history regarding insect species and divergent results in skin test and RAST (n=2)
- 3) One sting by an unknown insect together with positive skin test and/or positive RAST to both bee and vespid venom (n=4)
- 4) Several stings of partly unknown insects with positive skin test and/or positive RAST to both bee and vespid venom (n=4)

Controls had no history of systemic reactions to insect stings, determination of specific IgE-antibodies were negative. Skin tests were not performed for ethical reasons.

Basophil histamine release test (BHR)

300 μl heparinized whole blood was incubated with 300 μl honey bee venom (*Apis mellifera*) or yellow jacket venom (*Vespula spp.*) (Bühlmann, Allschwil, Switzerland) at different concentrations (0.0005, 0.005, 0.05 $\mu\text{g/ml}$) for 60 min at 37° C. Total histamine release was determined by incubation of the cells for 5 min at 100° C. As negative control 300 μl blood was incubated with buffer, 300 μl of anti-IgE diluted to 10^{-3} in the same buffer served as positive control. After 15 min in an ice

bath and centrifugation (500 g, 10 min, 4° C) supernatants were stored at -20° C. Afterwards histamine content in the supernatants was measured by an ELISA according to the manufacturer (IBL, Hamburg, Germany). The histamine release induced by hymenoptera venom was calculated as percentage of total histamine release (spontaneous release subtracted). Histamine release values > 10% were regarded positive.

Cellular Antigen Stimulation Test (CAST®)

2 ml whole EDTA blood were mixed with 0.5 ml of dextran solution and sedimented for 90 min at room temperature. The leukocyte containing upper phase was transferred into another tube and centrifuged for 15 min at 130 g and room temperature. The supernatant was discarded and the cells resuspended in 2 ml of stimulation buffer (containing IL-3). 75 μl of honey bee venom (*Apis mellifera*) or yellow jacket venom (*Vespula spp.*) at different concentrations (0.002, 0.02, 0.2 $\mu\text{g/ml}$), 75 μl of buffer (negative control) or of a monoclonal antibody to high affinity IgE receptor (stimulation control) were added to 300 μl cell suspension and incubated for 40 min at 37° C. All substances were purchased from the manufacturer (Bühlmann, Allschwil, Switzerland). After centrifugation for 3 min at 1000 g and 4° C the supernatants were stored at -20° C. Afterwards sulfidoleukotrienes (LTC₄, LTD₄, LTE₄) released into the supernatant were determined by an ELISA (CAST-ELISA) according to the manufacturer (Bühlmann, Allschwil, Switzerland). According to the instructions of the manufacturer results were regarded as positive, if > 681 pg/ml (bee venom) or 864 pg/ml (wasp venom) at 0.2 $\mu\text{g/ml}$ venom or > 607 pg/ml (bee venom) or 541 pg/ml (wasp venom) leukotrienes at 0.02 $\mu\text{g/ml}$ venom were released. Values for negative control (background) were subtracted.

Basophil activation test (BAT)

The BASOTEST® (Orpegen Pharma, Heidelberg, Germany) was used for the quantitative determination of in vitro basophil activation. 100 μl heparinized blood was first incubated with 20 μl stimulation buffer for 10 min at 37° C and then with 100 μl of allergen solution (bee or wasp venom diluted in buffer at a final concentration of 0.0045 $\mu\text{g/ml}$, 0.045 $\mu\text{g/ml}$, 0.45 $\mu\text{g/ml}$). 100 μl PBS solution (negative control) or 100 μl N-formyl-methionyl-leucyl-phenylalanine (FMLP) as positive control for 20 min at 37° C. The degranulation process was stopped by incubating the samples on ice for 5 min. 20 μl of phycoerythrin-conjugated anti-IgE and FITC-conjugated anti-gp53 were added and incubated for 20 min in an ice bath. Erythrocytes were destroyed by adding 2 ml lysing solution for 10 min at room temperature. Cells were washed twice with

washing solution and resuspended in 200 µl washing solution. Flow cytometric analysis was performed within 2 hours using a FACScan (Becton-Dickinson Immunocytometry System, Heidelberg, Germany) and CellQuest™ software. According to the instructions of the manufacturer the basophil population was gated by the presence of phycoerythrin-conjugated anti-IgE, and the expression of gp53 (CD63) was analysed on this gated cell population. Acquisition was performed on 1000 cells for each sample and results are given as the percentage of basophil expressing gp53. Results with more than 15% of activated basophils were regarded as positive according to the manufacturer.

Statistical analysis of the data

The concordance (i.e. positivity or negativity for both venoms) of the basophil activation test, histamine release test, cellular antigen stimulation test, skin test and determination of specific IgE antibodies, respectively were calculated. Correlations between BAT, BHR and CAST were analyzed using the Spearman correlation coefficient.

Results

Clinical characteristics and test results are shown in table 1.

History

Ten patients could not always define the insect, two patients remembered wasp stings, one patient a bee sting and one patient a hornet sting.

Skin tests

Two patients were skin test negative, one patient showed a questionable result for wasp venom (patient number 1), two patients had positive results only for bee venom, two only for wasp venom and seven for both.

Specific IgE-antibodies

Bee and wasp venom specific IgE-antibodies were quantified into CAP-classes. Four patients had no specific IgE-antibodies, two had antibodies only to bee venom, two only to wasp venom and six to both bee and wasp venom.

Basophil histamine release test (BHR)

Histamine release after incubation with wasp venom at

a concentration of 0.05 µg/ml was positive in 12 out of the 14 patients (85.7%), with bee venom in seven patients (50%). Maximum percent release of the individual patients is given in table 1. At a concentration of 0.005 µg/ml venom patient no. 1 showed a positive result (histamine release with wasp venom: 75.7%), at a concentration of 0.0005 µg/ml venom none (data not shown). Stimulation control with anti-IgE ranged from 1.4 to 75.9 ng/ml (mean±SD: 16.3±14.2 ng/ml), the negative control between 0.4 to 5.0 ng/ml (mean±SD: 1.78±1.01 ng/ml).

Cellular antigen stimulation test (CAST)

A positive result after incubation with wasp venom was found in 12 of the 14 patients (85.7%) at a concentration of 0.2 and 0.02 µg/ml, after incubation with bee venom in 6 patients (42.9%) at a concentration of 0.2 µg/ml and in 5 patients (35.7%) at a concentration of 0.02 µg/ml. Maximum leukotriene release (pg/ml) is shown in table 1. Stimulation control with anti-IgE ranged between 138 pg/ml and 6091 pg/ml (mean±SD: 2383.2±1542.9 pg/ml).

Basophil activation test (BAT)

More than 15% basophil activation after incubation with wasp venom was seen in 11 of the 14 patients (78.6%) at a concentration of 0.45 µg/ml, in 4 patients (28.6%) at a concentration of 0.045 µg/ml and one (patient no.6) at a concentration of 0.0045 µg/ml. With bee venom positive results were found in 7 patients (50%) at a concentration of 0.45 µg/ml, in 5 patients (35.7%) at a concentration of 0.045 µg/ml and none at a concentration of 0.0045 µg/ml. Details are given in table 1. Stimulation controls with FMLP ranged between 6.3 and 44.8% (mean±SD: 17.8±10.8%), negative controls between 0.4 and 9.4% (mean±SD: 5.8±3.1%). Controls were negative in all cases.

Concordance between In-vivo- and In-vitro-tests

Concordance of skin tests and cellular tests ranged between 35.7% (skin test vs. CAST) and 57.1% (skin test vs. BHR), of RAST and cellular tests between 42.9% (RAST vs. BHR) and 57.1% (RAST vs. CAST or BAT). The best concordance within the different cellular tests showed BAT and CAST with 78.6% (Table 2).

Correlations between the cellular tests

There was only some correlation between BAT and CAST with better results for bee venom. Other

Table 1. Clinical data of patients, results of in-vivo- and in-vitro-tests, indication for immunotherapy

No.	Sex	Age (years)	History (number of stings, kind of insect, severity grade [3])	Skin test negative - positive + questionable results (+)	Specific IgE antibodies (CAP-RAST class)	BHR release [%] at 0.05 µg/ml, positive > 10%	CAST release [µg/ml] at 0.02 µg/ml positive > 607 [B] > 541 [W]	BAT release [%] at 0.04 µg/ml positive > 15%	Indication for immunotherapy with	Comments regarding importance of BAT
1	m	67	6 x W, III	B - W (+)	B 0 W 0	B 13.3 W 100.0	B 83 W 3723	B 2.8 W 43.4	W	BAT confirms results of CAST, not of BHR
2	f	28	> 1 x unclear, I x II	B + W +	B 0 W 1	B 49.3 W 39.3	B 0 W 4325	B 1.5 W 58.0	W	BAT confirms results of CAST, not of BHR
3	m	41	> 1 x B, II	B - W +	B 1 W 4	B 7.6 W 15.3	B 0 W 565	B 4.6 W 8.6	W	Concordance of BAT, BHR and CAST
4	f	39	1 x unclear, II	B + W +	B 0 W 0	B 7.4 W 6.3	B 139 W 153	B 8.8 W 6.3	B	Only BAT gives a positive result
5	m	38	> 1 x unclear, I x W, I	B + W +	B 2 W 0	B 47.9 W 21.3	B 6338 W 2277	B 53.2 W 6.8	B and W	Concordance of BAT BHR and CAST
6	f	32	1 x unclear, II	B + W -	B 0 W 1	B 1.6 W 45.7	B 0 W 6438	B 5.6 W 71.4	W	Concordance of BAT BHR and CAST
7	m	58	1 x W, II	B + W +	B 3 W 4	B 7.7 W 31.6	B 119 W 5353	B 5.3 W 15.4	W	Concordance of BAT BHR and CAST
8	f	12	1 x unclear, I	B + W -	B 6 W 3	B 100.0 W 37.7	B 1047 W 5080	B 36.1 W 6.1	B	Concordance of BAT BHR and CAST
9	m	65	1 x W, I 1 x unclear, III	B + W +	B 2 W 3	B 18.7 W 100.0	B 1089 W 1836	B 9.9 W 12.2	B and W	Concordance of BAT BHR and CAST
10	m	34	1 x hornet, II	B + W +	B 0 W 0	B 44.3 W 72.9	B 258 W 1857	B 33.1 W 8.8	W	Unchanged decision for IT by BAT
11	m	31	1 x unclear, III 1 x W, II	B - W -	B 1 W 0	B 8.9 W 26.8	B 1099 W 1445	B 31.2 W 2.6	B and W	Unchanged decision for IT by BAT
12	m	58	1 x unclear, III	B - W +	B 2 W 3	B 0 W 34.1	B 211 W 3392	B 6.5 W 85.1	W	Unchanged decision for IT by BAT
13	m	52	2 x unclear, I	B + W +	B 3 W 1	B 19.1 W 11.7	B 7436 W 7133	B 43.5 W 9.5	B and W	Concordance of BAT BHR and CAST
14	m	40	1 x unclear, II	B - W -	B 0 W 0	B 0 W 0	B 147 W 290	B 5.1 W 1.2	no ind. for IT	Concordance of BAT BHR and CAST

BHR. Basophil histamine release. CAST. Cellular antigen stimulation test. BAT. Basophil activation test.
f. Female. m. Male. B. (Reaction to) bee (venom). W (Reaction to) wasp (venom). † = over detection limit. ind. Indication. IT. Immunotherapy. **BOLD = positive results**

Table 2. Concordance of results obtained by different test systems

Test systems	Concordance
Skin test, RAST vs. cellular tests	
Skin test vs. BHR	57.1%
Skin test vs. CAST	35.7%
Skin test vs. BAT	42.9%
CAP-RAST vs. BHR	42.9%
CAP-RAST vs. CAST	57.1%
CAP-RAST vs. BAT	57.1%
Cellular tests	
BHR vs. CAST	64.3%
BAT vs. CAST	78.6%
BAT vs. BHR	64.3%
BAT vs. CAST vs. BHR	57.1%

BHR. Basophil histamine release test. CAST. Cellular antigen stimulation test. BAT. Basophil activation test.

Table 3. Correlation between basophil activation test (BAT) (0.45 µg/ml venom), basophil histamine release (BHR) (0.05 µg/ml) and cellular antigen stimulation test (CAST) (0.02 µg/ml).

Test systems	r
BAT vs. BHR (bee venom)	0.29
BAT vs. BHR (wasp venom)	-0.42
BHR vs. CAST (bee venom)	0.25
BHR vs. CAST (wasp venom)	0.40
BAT vs. CAST (bee venom)	0.60
BAT vs. CAST (wasp venom)	0.40

BHR. Basophil histamine release test. CAST. Cellular antigen stimulation test. BAT. Basophil activation test

correlations were weak or not existent (Table 3). A correlation between BAT and CAST at a concentration of 0.2 µg/ml could not be calculated, because in 3 cases the results were over the detection limit (Table 1).

Selection of insect venom species for specific immunotherapy

In 8 (No. 3,5,6,7,8,9,13,14) of the 14 patients BAT, BHR and CAST were in concordance. Therefore the decision for immunotherapy was unchanged by the introduction of BAT. In the remaining cases BAT did not add further information in three cases (No. 10,11,12), but was decisive for the selection in 3 cases (No. 1,2,4, see comments in table 1). The results of BAT did not complicate the decision in any case.

Discussion

This study shows that the flowcytometric measurement of CD63 expression after stimulation with bee and wasp venom as marker of basophil activation is useful as additional cellular test in the diagnosis of hymenoptera venom allergy. Such sophisticated and expensive diagnostic tests are usually not performed for routine clinical use. They are used for experimental studies or recommended in cases, where the decision as to the selection of the relevant insect species is difficult either because hymenoptera-specific IgE-antibodies cannot be found or history, skin test and determination of specific IgE-antibodies show contradictory results [4].

BHR was already used in the seventies by the group of Lichtenstein in order to characterize allergenic components of insect venoms [5,6] and as diagnostic method in a few patients [7]. Later, in a large group of insect venom allergic patients (n=181) a specificity of 94% and a sensitivity of 82% for bee venom-induced histamine release and a specificity of 83% and a sensitivity of 68% for wasp-venom-induced histamine release was found [8]. In this study peripheral blood leukocytes were washed and histamine was determined fluorometrically by the method of Siraganian [9]. In another study with whole blood histamine release, which we also used, these values were worse with a sensitivity of the histamine release assay of 62.5% for bee venom and 50% for wasp venom and a specificity of 44% for bee venom and 60% for wasp venom [10].

Measurement of de novo synthesized sulfidoleukotrienes (LTC₄, LTD₄, LTE₄) after allergen stimulation in a commercially available test (CAST) was introduced in 1993 by de Weck et al. [11]. Studies of patients with wasp venom allergy with this test showed a concordance of CAST to RAST in 84% and of CAST to skin test in 88% [12]. As expected, in our special study group these values were lower (Table 2). Correlation coefficients between sulfidoleukotriene generation and histamine release were found to be -0.02 for bee venom and 0.13 for wasp venom [10]. In our study the corresponding values were 0.20 and 0.13 (Table 3). These data suggest that these mediator responses do not occur in parallel. Some studies [12,13] showed positive results with the CAST after incubation with wasp venom at higher concentrations in several controls.

The concept of flowcytometric measurement of CD63 expression as marker of basophil activation was published in 1991. The CD63 marker is a 53-kDa glycoprotein present on the lysosome membrane and is expressed with a high density on activated basophil membrane [14]. Several investigators assessed the usefulness of this marker after stimulation of the cells with anti-IgE, FMLP or allergens in experimental and clinical studies with favourable results [14-24]. Also in patients with insect venom allergy incubation of basophils with hymenoptera venom induced an upregulation of CD63 [25-27], but not an increased

expression of CD63 without incubation with allergen [15].

The correlation of BAT with BHR in our study was between 0.25 and 0.37 and between BAT and CAST between 0.54 and 0.84 in dependence of the kind of venom (Table 3). In a recently published study about the diagnosis of insect venom allergy comparing flowcytometry, histamine and leukotriene C4 release [27] the values were higher for the histamine release ($r=0.61$) and lower for the leukotriene C4 release ($r=0.38$), but methods for all three tests and positivity thresholds were different to our study.

In the above mentioned study flowcytometry, histamine and leukotrienes C4 release showed a sensitivity of 100%, 89% and 100%, respectively, compared to the clinical history [28]. However, it should be mentioned that the history of the patients included local reactions, which are not an indication for immunotherapy [1,4]. In our study we put emphasis on the clinical usefulness of the tests with regard to the indication for immunotherapy in difficult individual cases of hymenoptera venom allergy. In our opinion the decision for immunotherapy with the relevant insect was facilitated, if two cellular tests – CAST and BAT were performed (Table 1). All studies including our study using BAT with hymenoptera venom showed negative results in controls [27,28], which also argues for the performance of this test in clinical diagnosis. More experience is needed, but BAT should be considered as a useful additional method for the diagnosis of hymenoptera venom allergy.

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