Original Article

In vitro basophil activation using CD63 expression in patients with bee and wasp venom allergy

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Summary. 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. Cellular tests can add useful information but the role of basophil activation tests for the different venoms has to be elucidated further.
We evaluated positive reactions in a basophil activation test using CD63 expression as marker independently for bee or wasp venom in patients with *hymenoptera* allergy.
Fifty-seven patients with a history of insect venom anaphylaxis were examined (12 x bee venom, 39 x wasp venom, 6 x bee plus wasp venom). Skin tests and determination of specific IgE-antibodies were performed. Basophil activation test (BAT) using CD63 expression was performed after stimulation with 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.
In patients with bee venom allergy, BAT was positive in 100% to bee venom and 75% to wasp venom. In patients with bee and wasp venom allergy, positive reactions for both venoms were found in 100%. In patients with wasp venom allergy, 97% reacted positive to wasp venom and only 56% to bee venom.
These results show the reliability of the basophil activation test as a cellular test in the *in vitro* diagnosis in patients with bee and wasp venom allergy. They also show that positive reactions in the basophil activation test reflect both sensitization status and cross-reactivity between venom species.

Key words: basophils, basophil activation test, flow cytometry, CD63, bee and wasp venom allergy.

Resumen. El diagnóstico de la alergia al veneno de insectos y la indicación de una inmunoterapia específica se basan en la historia clínica, las pruebas cutáneas y la presencia de anticuerpos IgE específicos frente al veneno de himenópteros. Las pruebas celulares pueden aportar información útil, aunque el papel de los tests de activación de basófilos para los diferentes venenos todavía no está del todo claro.
Se evaluaron las reacciones positivas en el test de activación de basófilos utilizando la expresión de CD63 como marcador, independientemente para veneno de abeja y de avispa, en pacientes con alergia a himenópteros. Se examinaron 57 pacientes con antecedentes de anafilaxia al veneno de insectos (12 para el veneno de abeja, 39 para el veneno de avispa, 6 para el veneno de abeja y avispa). Se realizaron pruebas cutáneas y se determinaron los anticuerpos IgE específicos. El test de activación de basófilos (TAB) se llevó a cabo utilizando la expresión de CD63 tras la estimulación con diferentes concentraciones de veneno de abeja y avispa. El TAB se basa en una doble tinción con anticuerpos anti-IgE y anti-CD63 y la posterior determinación del porcentaje de basófilos activados mediante citometría de flujo. En pacientes con alergia al veneno de abeja, el TAB fue positivo en un 100% al veneno de abeja y en un 75% al veneno de avispa. En pacientes con alergia al veneno de abeja y avispa, se registró un 100% de reacciones positivas para ambos venenos. En pacientes con alergia al veneno de avispa, un 97% reaccionaron de forma positiva al veneno de avispa y sólo un 56% al veneno de abeja. Estos resultados muestran la fiabilidad del test de activación de basófilos como prueba celular en el diagnóstico *in vitro* en pacientes con alergia al veneno de abeja y avispa. También muestran que las reacciones positivas en el test de activación de basófilos reflejan tanto el estado de sensibilización como la reactividad cruzada entre las especies con veneno.

Palabras clave: Basófilos, test de activación de basófilos, citometría de flujo, CD63, alergia al veneno de abeja y avispa.

Table 1. Clinical characteristics of patients

History of anaphylactic reaction to	n	Males (n)	Females (n)	Age (years)	Mean age and SD (years)	Severity grade of last reaction I (n)	II (n)	III (n)
Bee sting	12	6	6	10-70	42.3±20.3	2	7	3
Bee and wasp sting	6	5	1	29-68	45.7±15.3	1	5	0
Wasp sting	39	18	21	13-79	45.7±16.1	8	23	8
Total	57	29	28	10-79	44.8±16.5	11	35	11

Introduction

Life-threatening anaphylactic reactions to bee or vespid stings occur in 0.8-5% of the general population [1]. Treatment of this IgE-mediated allergy with specific immunotherapy is highly effective: complete protection, namely the absence of systemic clinical symptoms in sting challenge, has been found in 80-100% [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. Additional cellular tests like histamine release or cellular antigen stimulation test [3,4] are recommended in unclear cases, where the decision regarding the relevant insect species for immunotherapy is difficult. Moreover, the basophil activation test using CD63 expression as marker [5] has been used in these situations [6]. Sensitivity of this test varied between 85% and 100% and specificity between 83% and 100%. Here, we assessed the reactivity in the basophil activation test using CD63 expression on basophils as marker, separately for bee and wasp venom, in 57 patients with *hymenoptera* venom anaphylaxis.

Material and methods

Patients

A total of 57 consecutive patients with insect venom anaphylaxis were evaluated before immunotherapy: 12 patients (6 male, 6 female; age: 10 to 70 years, mean: 42.3±20.3 years) with a history of systemic reactions to bee venom, 39 patients (18 male, 21 female; age: 13-79 years; mean 45.7±16.1 years) with a history of systemic reactions to wasp venom and 6 patients (5 male, 1 female; age: 29-68 years; mean 45.7±15.3 years) with a history of systemic reactions to bee and wasp venom, as well as 10 healthy controls (5 male, 5 female; age: 37-67 years; mean 50.1±12.3 years) with negative history and absence of specific IgE.

Intradermal skin testing with honeybee or yellow jacket venom (Venomil; Bencard, Munich, Germany) was carried out on the ventral aspect of the forearm with incremental concentrations of 0.0001, 0.001, 0.01 and 0.1 µg/ml with a positive (histamine dihydrochloride) and negative control (saline). Positive test results were defined according to the recommendations of the European Academy of Allergology and Clinical Immunology (EAACI) [7]. Venom-specific serum IgE-antibodies were determined by a fluorescence enzyme immunoassay (Pharmacia CAP RAST FEIA, Uppsala, Sweden) (Table 1-4).

Table 2. Results of skin test, immunoglobulin E (IgE) detection and basophil activation test in patients with a history of anaphylactic reactions to bee stings (n=12).

Venom	Skin Positive (n)	Venom specific IgE-antibodies (RAST-class: n) 0	1	2	3	4	5	6	Basophil activation test positive (n) at an allergen solution of 1 µg/ml	0.1 µg/ml	0.01 µg/ml
Bee venom	12	1	0	2	2	5	2	0	12	10	6
Wasp venom	6	4	0	5	3	0	0	0	8	6	1

Table 3. Results of skin test, immunoglobulin E (IgE) detection and basophil activation test in patients with a history of anaphylactic reactions to bee and wasp stings (n=6).

Venom	Skin test positive (n)	Venom specific IgE-antibodies (RAST-class: n) 0	1	2	3	4	5	6	Basophil activation test positive (n) at an allergen solution of 1 µg/ml	0.1 µg/ml	0.01 µg/ml
Bee venom	6	0	0	1	0	3	1	1	6	5	4
Wasp venom	6	0	0	1	2	2	0	1	6	5	4

Table 4. Results of skin test, immunoglobulin E (IgE) detection and basophil activation test in patients with a history of anaphylactic reactions to wasp stings (n = 39).

Venom	Skin test positive (n)	Venom specific IgE-antibodies (RAST-class: n) 0	1	2	3	4	5	6	Basophil activation test positive (n) at an allergen solution of 1 µg/ml	0.1 µg/ml	0.01 µg/ml
Bee venom	11	18	2	8	10	1	0	0	23	7	3
Wasp venom	39	1	3	12	17	3	1	2	38	37	19

Basophil activation test (BAT)

The BASOTEST® (Orpegen Pharma, Heidelberg, Germany) was used for the quantitative determination of *in vitro* basophil activation. 100 µl heparinized whole 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; Reless®, ALK SCHERAX, Hamburg, Germany) diluted in buffer at a final concentration of 1 µg/ml, 0.1 µg/ml and 0.01 µg/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 (Becton-Dickinson) 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 TM 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 and a previous published study based on ROC analysis [8]. Negative controls were below this value.

Statistical analysis

Results of the basophil activation test are expressed as mean and standard deviation of the percentage of CD63+ basophils.

Results

Clinical characterisation of patients

Of the 12 patients with a history of anaphylactic reactions to bee stings, 2 experienced a grade I reaction, 7 a grade II reaction and 3 a grade III reaction. Reactions were graded according to Ring and Messmer [9]. Skin test to bee venom was positive in 12 cases and to wasp venom in 6 cases. All except one showed bee venom-specific serum IgE-antibodies, 8 wasp venom antibodies. Of the 6 patients with a history of anaphylactic reactions to bee and wasp stings, 1 experienced a grade I reaction and 5 a grade II reaction. Skin test to bee and wasp venom was positive in all cases. Specific serum IgE-antibodies to both venoms were demonstrable in all patients. Of the 39 patients with a history of anaphylactic reactions to wasp stings, 8 experienced a grade I reaction, 23 a grade II reaction and 8 a grade III reaction. Skin test to bee venom was positive in 11 cases and to wasp venom in 38 cases. All except one showed wasp venom-specific serum IgE-antibodies and 21 bee venom antibodies. Controls had a negative history and a negative RAST for both venoms. (Table 1 - 4)

Basophil activation test

In 12 bee venom allergic patients, basophil activation test was positive in all cases to bee venom at the highest concentration, and in 8 cases to wasp venom at the highest concentration. In 6 patients with a history of anaphylactic reactions to bee and wasp stings, basophil activation test was positive in all cases for wasp and bee venom. In 39 patients with wasp venom allergy, 38 showed a positive basophil activation test to wasp venom at the highest concentration and 23 to bee venom at the highest concentration. Lower venom concentrations resulted in less frequent positive reactions (Table 2,3,4). The mean percentage of basophil activation for both venoms at various concentrations in the different patient groups are

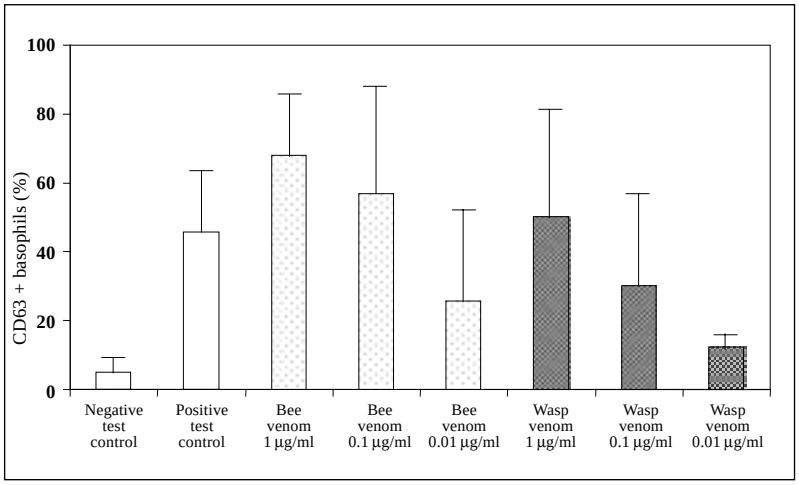

Figure 1. CD63 expression of basophils after incubation with buffer (negative control), FMLP (positive control) and three bee or wasp venom solutions in patients with a history of anaphylactic reaction to bee stings (mean ± standard deviation; n=12).

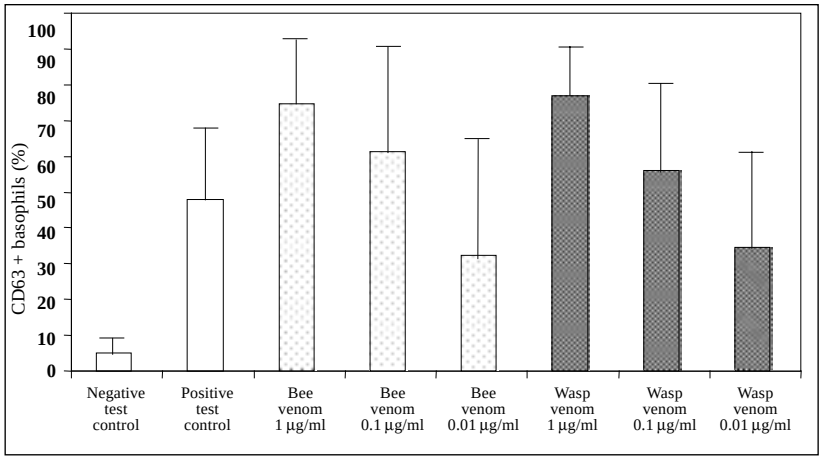

Figure 2. CD63 expression of basophils after incubation with buffer (negative control), FMLP (positive control) and three bee or wasp venom solutions in patients with a history of anaphylactic reaction to bee and wasp stings (mean ± standard deviation; n=6).

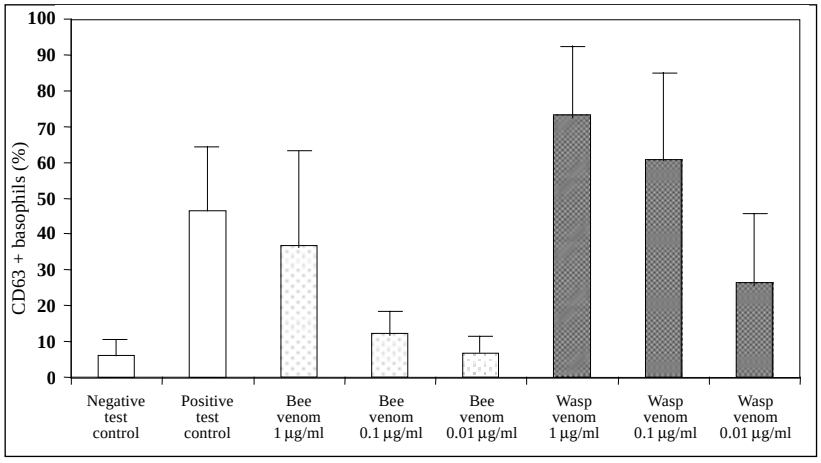

Figure 3. CD63 expression of basophils after incubation with buffer (negative control), FMLP (positive control) and three bee or wasp venom solutions in patients with a history of anaphylactic reaction to wasp stings (mean ± standard deviation; n=39).

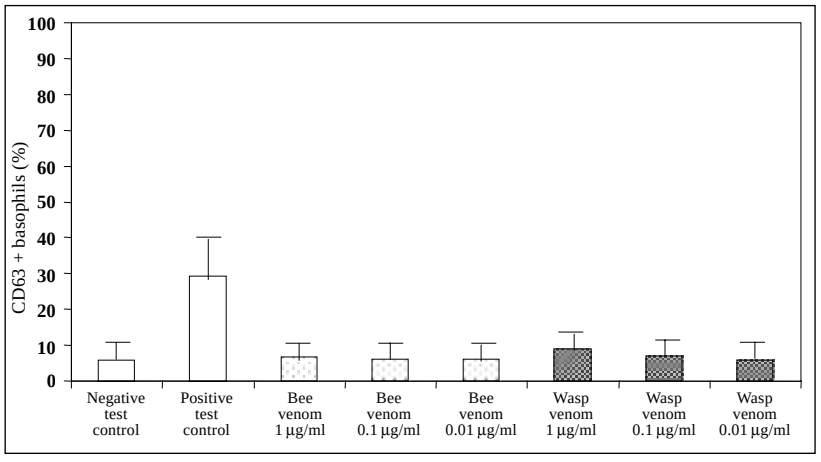

Figure 4. CD63 expression of basophils after incubation with buffer (negative control), FMLP (positive control) and three bee or wasp venom solutions in controls with a negative history of anaphylactic reaction to hymenoptera stings and failure to detect specific IgE-antibodies (mean ± standard deviation; n=10).

shown in Figs. 1-4. Basophil activation at the highest concentrations of the relevant insect venom did not correlate with the clinical severity as shown by history: Basophil activation (mean and ± standard deviation) at grade I was 76.5±16.9% (n=12), at grade II 69.7±20.5% (n=40) and at grade III 77.9±16.1% (n=11).

All controls were negative in the BAT.

Discussion

This study shows that the basophil activation test with CD63 is a sensitive and reliable cellular *in vitro* test in the diagnosis of bee and wasp venom allergy: Positive reactions for the insect responsible for the anaphylactic reaction varied between 97% and 100% in the basophil

activation test, between 92% and 100% in the RAST and the skin test was positive in 100%. These data are in good concordance with other studies using the same method for basophil activation: In patients with wasp venom allergy, sensitivity of BAT was 92% or 100%, respectively [8,10]. For patients with a bee venom allergy or a bee and wasp venom allergy, comparable data with exactly the same test system are not available. Using a basophil activation test without pre-incubation with IL-3, 91.3% tested positive in the basophil activation test for bee venom and 85.3% for wasp venom [11], suggesting that pre-incubation with IL-3 increases sensitivity. Overall sensitivity was 100% in another study, although patients with local reactions to hymenoptera sting were also included [12].

In previous studies using basophil activation tests, specificity varied between 80% and 100%, but part of the controls also had elevated specific IgE, which was not the case in our study: In one study, all eight control subjects (out of 30) who tested positive in the basophil activation test also had elevated specific IgE [11], in another study three of 20 controls showed specific IgE to wasp and two of 20 to bee venom [8]. These data are in accordance with results of a study in a general population cohort. In 27.1% of the sera, specific IgE antibodies to *hymenoptera* venom were found. Only 7.1% of them reported systemic anaphylactic reactions [13]. We defined controls as indivuals with negative history and failure to detect specific IgE-antibodies. In such cases, the basophil activation test was negative for both venoms.

These results and the results of our study clearly show that positive reactions to the BAT reflect the status of sensitization, proved by skin test or RAST, without correlation to the clinical severity as shown in history. This is underlined by the fact that the basophil activation test is not an alternative to the sting challenge in monitoring successful immunotherapy, and not a parameter to predict the individual risk for severe anaphylactic reactions [8,11]. Furthermore, CD63 expression of activated basophils increased when studied 1 week after venom immunotherapy [14] and did not decrease significantly during long-term immunotherapy [8, 10].

Generally, the CD63 basophil activation test showed better results for sensitivity and specificity when compared with histamine release assays and better results for specificity when compared with leukotriene release assays [3,11,12,15-19]. This might be the main advantage of the basophil activation test as an additional cellular test for IgE-mediated insect allergy. As published previously, we and others could show that the basophil activation test is very useful in difficult cases of *hymenoptera* allergy, where history, skin test and determination for specific antibodies are discrepant and do not allow a clear decision regarding the relevant insect species for immunotherapy. In special cases, sensitization to *hymenoptera* venoms was only demonstrated by the basophil activation test giving the crucial clue for the selection of the appropriate venom for immunotherapy [10,18].

The results of the study also reveal the problem of double sensitization to honey bee and wasp venom, which was shown in about 30% of the *hymenoptera*-allergic patients by determination of specific IgE-antibodies [20]. The basophil activation test does not seem to be capable of identifying cross-reactors. Additional information, such as inhibition data, are still needed.

Taken together, the CD63 basophil activation test should be considered as a useful additional *in vitro* method for the diagnosis of *hymenoptera* venom allergy with its limitations, but it also has clear advantages compared to other cellular tests based on the *in vitro* reaction of blood basophils in the presence of the putative allergen [21,22].

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