

Original article

The basophil activation test in wasp venom allergy: sensitivity, specificity and monitoring specific immunotherapy

Background: As *in vitro* diagnosis of wasp venom sensitization by specific serum IgE has a sensitivity of only 60–80%, additional *in vitro* tests are desirable. Basophil activation is associated with the expression of CD63 and its measurement has been proposed as a novel *in vitro* test for immediate-type allergy. Furthermore, to date, no *in vitro* test exists to monitor successful specific immunotherapy (SIT) with wasp venom. Therefore, the potentially harmful sting challenge is still recommended.

Objective: We compared the CD63-based basophil activation test (BAT) in the diagnosis of wasp venom allergy with skin tests and measurement of specific IgE. Furthermore, we investigated whether BAT can predict the outcome of the sting challenge in patients on SIT.

Methods: Fifty patients with a systemic reaction caused by a wasp sting and 20 controls were studied. Intracutaneous tests were performed with wasp and bee venom in the suspected allergics. Specific IgE was determined by the CAP-FEIA method and basophil activation by flow cytometry upon double staining with anti-IgE/anti-CD63 mAb. Twenty-five patients were sting challenged 6 months after starting SIT and the BAT was repeated before challenge.

Results: Sensitivity of the intracutaneous tests, specific IgE and BAT was 100, 76, and 92%, respectively. Specificity of specific IgE and the BAT was 85 and 80%, respectively. The cut-off for a positive BAT was 15% CD63+ basophils. There was a positive correlation between IgE reactivity to wasp venom and the number of CD63+ basophils ($r = 0.65$). Although no patient had a systemic reaction upon sting challenge, in most subjects basophil activation did not decrease when compared with the BAT before SIT.

Conclusions: Quantitation of basophil activation by CD63 expression is a valuable new *in vitro* method for diagnosis of allergy to hymenopteran venoms. The CD63-based BAT is a helpful tool for the complementation of routine diagnostic tests such as specific IgE as it increases sensitivity of *in vitro* detection of sensitization. However, this *in vitro* method does not offer an alternative to the sting challenge in monitoring successful SIT.

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It is estimated that up to 5% of the general population suffer from potentially life-threatening systemic reactions after hymenopteran stings (1). The diagnostic work-up of wasp and bee venom allergy and treatment by specific venom immunotherapy (VIT) is well established and suspected allergy is most often confirmed by skin testing. However, to date, *in vitro* diagnosis by specific IgE has still a diagnostic gap of up to 20–30% particularly when performed more than 1 year after the last sting reaction (1). Therefore, additional *in vitro* methods are desirable to make *in vitro* testing as reliable as *in vivo* testing. Secondly, despite great progress in understanding the mechanism of VIT, there is still no *in vitro* test which predicts whether a patient has been successfully treated

by VIT, i.e. whether a patient will or will not have a systemic reaction in case of a future sting. Therefore, the potentially life-threatening sting challenge is still recommended (2).

Basophils are often used as target cells for *in vitro* assays to detect IgE-mediated sensitization. It has been demonstrated that the surface marker CD63 is expressed with high density on activated basophils yet only weakly on resting cells (3). The CD63 glycoprotein, which is present on the membrane of cytoplasmic granules, is expressed only to an extent of <5% on the outside membrane of resting basophils, but with high density on activated basophils after the fusion of these granules with the cytoplasmic membrane (3–5). Flow

cytometric detection of basophil activation by CD63 expression has been studied in detection of sensitization in immediate type allergy to various allergens such as natural rubber latex (6, 7), foods (8), aeroallergens (9, 10), hymenopteran venoms (11) and drugs (12). This new method has been commonly referred to as the basophil activation test (BAT). However, to date, validation of the BAT for diagnosis of wasp venom allergy is still limited and no study exists on wasp allergic subjects on VIT including sting challenges to assess success of treatment.

The aim of this study was to investigate whether a two-colour flow cytometric method using phycoerythrin-conjugated anti-IgE and fluorescein isothiocyanate-conjugated anti-CD63 monoclonal antibodies (mAb) to analyse human basophil activation can be applied to diagnose wasp venom allergy and to compare its sensitivity and specificity to well-established routine tests such as intracutaneous skin testing and measurement of allergen-specific serum IgE. A second aim was to investigate whether the BAT can predict the outcome of sting challenges in patients replacing this potentially dangerous procedure in monitoring VIT.

Material and methods

Patients

Fifty patients (18–58 years old, mean 44 years) with a most suggestive history of a systemic adverse reaction such as urticaria, angioedema, dyspnoea, or anaphylactic shock caused by a wasp sting were selected for this study. Forty-one of 50 patients had had an adverse systemic reaction within 1 year before referral to our department, in nine patients, the adverse reaction was more than 1 year ago. Although 15 of 50 patients had also experienced bee stings, no systemic reactions to bee stings had occurred. Twenty healthy subjects (19–60 years old, mean 40 years) with no history of an adverse reaction to wasp or bee stings served as controls. The suspected wasp allergics were classified according to the severity of the systemic reaction according to the classification by H. L. Müller which encompasses four classes (13). Thirty of 50 (60%) patients were classified Müller grade 2, 15 of 50 (30%) grade 3, and five of 50 (10%) grade 4.

Skin testing

Intracutaneous skin testing was performed on the flexor aspect of the forearm with wasp and bee venom with allergen concentrations ranging from 10^{-7} – 10^0 µg/ml (Bencard, Munich, Germany) and with a positive (histamine dihydrochloride) and a negative control (diluent). In the controls, no *in vivo* but only *in vitro* testing was performed for ethical reasons. We recorded reactions according to the recommendations of the European Academy of Allergology and Clinical Immunology (EAACI) (14).

Testing for IgE

Total IgE and specific IgE to wasp and bee venom were measured by using the CAP FEIA system according to the recommendations of the manufacturer (Pharmacia & Upjohn, Uppsala, Sweden).

Basophil CD63 surface expression

Peripheral blood samples from all subjects were drawn into heparinized tubes; 100 µl of whole blood was pre-incubated with 20 µl of wash buffer [containing interleukin (IL)-3 at a final concentration of 2 ng/ml] at 37°C for 10 min; 100 µl of the cell suspension was distributed in microtiter plate wells and mixed in parallel with 100 µl of wash buffer (negative control), 100 µl of N-formyl-methionyl-leucyl-phenylalanine (fMLP) (1 µmol/l as positive control) and wasp and bee venom in a volume of 100 µl each. One hundred nanograms and 1 µg wasp venom and 100 ng and 1 µg bee venom in duplicate (Bencard). Before this study, the BAT had been performed in a pilot study in seven wasp and seven bee allergics with wasp and bee venom in seven increasing dilutions (10 µg– 10^{-4} µg). A bell-shaped dose-response curve was demonstrated for both allergens (Fig. 1). Incubating with allergen extracts within a range of 100 ng to 2 µg yielded best results with regard to mean basophil activation (Fig. 1). No basophil activation was induced in controls. These results are in keeping with studies of other groups who demonstrated that optimal basophil activation is induced by a wide range of allergen concentrations (15).

Plates were incubated for 20 min at 37°C. Degranulation was stopped by chilling on ice for 5 min. Thereafter, to all samples phycoerythrin-conjugated anti-IgE (PE anti-IgE) and fluorescein isothiocyanate (FITC)-conjugated anti-CD63 mAb were added and incubated for 20 min on ice. Finally, the heparinized whole blood probes were lysed, fixed, and washed. Plates were centrifuged ($250 \times g$, 5 min, 4°C) and analysed within 2 h on a FACScan flow cytometer (Becton Dickinson Immunocytometry System, San Jose, CA, USA). IL-3, fMLP, PE anti-IgE and FITC-conjugated anti-CD63 mAb were purchased from Orpegen Pharma (Heidelberg, Germany). The basophil population was gated by the expression of PE anti-IgE. The expression of CD63 was analysed on this gated cell population. An average of 400 IgE-expressing basophils per probe were acquired and results were given as percentage of basophils expressing CD63 according to the following formula: number of PE anti-IgE + and FITC anti-CD63 + basophils/number of PE anti-IgE + basophils.

In the 25 patients who consented to the sting challenge, the CD63-based BAT was performed twice and results were compared: before starting VIT and 6 months later prior to the sting challenge.

Venom immunotherapy

In all 50 patients with confirmed IgE-mediated allergy to wasp venom, VIT with wasp venom (Bencard) was started according to the

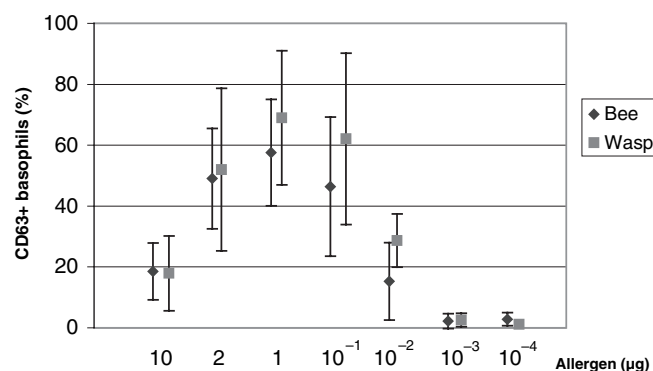


Figure 1. CD63 expression on basophils in a dose-response curve after incubation with wasp or bee venom in seven venom doses (10 – 10^{-4} µg) in wasp ($n = 7$) and bee allergics ($n = 7$).

position paper of the EAACI (1). VIT was started according to a modified rush protocol reaching the maintenance dose of 100 µg wasp venom within 5 days (1). The maintenance dose was finally administered at intervals of 4 weeks.

After 6 months of treatment with wasp venom, we recommended a sting challenge to all patients to assess successful treatment (2); 25 of 50 patients consented to a sting challenge by a wasp which was performed under complete resuscitation equipment.

Statistical analysis

Data of BAT are expressed as mean and standard deviation of the percentage of CD63+ basophils. The Fisher test was applied to compare the sensitivity and specificity of the CD63-based BAT and specific IgE and to prove whether BAT is more sensitive than measurement of specific IgE.

Receiver-operating characteristic (ROC) curves were used to assess the optimal cut-off (percentage of CD63+ basophils) for a positive BAT. An ROC curve is a plot of a test's sensitivity (on the y-axis) vs. its false-positive (FP) rate (i.e. 1-specificity, plotted on the x-axis) for different decision thresholds for defining positive and negative results (16). Sensitivity and specificity were calculated according to the method of Goldman (17) as follows: in this study a true-positive (TP) result was defined as a patient with a positive history of a wasp allergy and a positive skin test, or CAP or BAT; a FP result was defined as a patient with a negative history of a wasp allergy and a positive skin test, or CAP, or BAT; a true-negative (TN) result was defined as a patient with a negative history of a wasp allergy and a negative skin test, or CAP or BAT; a false-negative (FN) result was defined as a patient with a positive history of a wasp allergy and a negative skin test, or CAP or BAT.

$$\text{Sensitivity} = \frac{\text{TP}}{\text{TP} + \text{FN}}$$

$$\text{Specificity} = \frac{\text{TN}}{\text{TN} + \text{FP}}$$

The Welch test was applied to demonstrate whether the BAT is able to prove sensitization when compared with controls. Furthermore, the results of the BAT were compared with measurement of allergen-specific IgE by applying the Spearman's correlation coefficient for both allergen doses (0.1 and 1.0 µg) of wasp and bee venom. Spearman's correlation coefficient was also applied to check whether the BAT before the sting challenge is a useful method to monitor VIT, i.e. whether there is a correlation between the results before VIT and 6 months after starting VIT prior to the sting challenge.

The Spearman's correlation coefficient measures the degree of monotony between two (at least ordinal valued) random variables. In this study, the Spearman's correlation coefficient of specific IgE and the BAT measures the degree of monotony or the degree of equivalence between specific IgE and BAT, respectively. A value close to plus or minus 1 indicates equivalence, whereas a value close to 0 does not support equivalence.

Results

Immunotherapy

None of the 25 patients who were sting challenged by a wasp showed a systemic adverse reaction.

Skin testing

In all 50 patients with a most suggestive history of a systemic reaction caused by a wasp sting, wasp venom allergy could be confirmed by skin testing with wasp venom (sensitivity: 100%). In eight of 50 patients, skin testing with bee venom also showed sensitization to bee venom. Specificity could not be calculated, as in the controls, no skin testing was performed.

Testing for IgE

Mean total IgE in the group of allergics was 90 kU/l (range: 4–340 kU/l) and in the controls 82 kU/l (range: 6–430 kU/l). In 38 of 50 suspected wasp allergics there was proof of specific IgE to wasp venom giving a sensitivity 76% (95% CI: 62–87%). The 38 wasp allergics were classified as follows with regard to wasp venom CAP class: 15 CAP class 2, 19 CAP class 3, and four CAP class 4. In 12 patients, there was also proof of specific IgE to bee venom: seven CAP class 2, five CAP class 3.

Despite selection of the 20 controls by clinical history ('no adverse reaction to a wasp or a bee sting') three of 20 controls were sensitized to wasp and two of 20 to bee venom reducing specificity of diagnosing wasp venom allergy to 85% (95% CI: 62–97%).

Basophil CD63 surface expression

In 50 wasp allergics, the mean percentage of CD63+ basophils (of the total of all IgE+ basophils) after incubating the basophils with wash buffer (negative control) was 3.8% (SD: 2.63%) when compared with an average activation of 4.88% of all basophils (SD: 2.78%) in the 20 controls. Incubating the basophils with fMLP (positive control) resulted in a mean basophil activation of 44.61% (SD: 24.52%) in the wasp allergics and a mean basophil activation of 46.48% in the controls (SD: 22.57%).

A mean CD63 expression of basophils ranging between 3.8 and 4.88% before basophil activation either by the positive control or relevant allergen extracts implies that choosing an appropriate cut-off for a positive BAT has to reflect this basal CD63 expression ('background'). In most former studies, a cut-off for a positive BAT was only arbitrarily set at a percentage of CD63+ basophils exceeding the mean CD63 expression without stimulation ('background') by two standard deviations or a stimulation index was calculated as the ratio between the basal CD63 expression ('background') and the allergen induced basophil activation (12). In this study, calculating a cut-off on the basis of the basal CD63 expression without stimulation (negative control) plus 2 standard deviations would imply a cut-off for a positive BAT at approximately 12% CD63+ basophils. As described in this section (below) ROC analysis also suggests a cut-off exceeding 10% CD63+ basophils. In the group of wasp allergics, incubating the

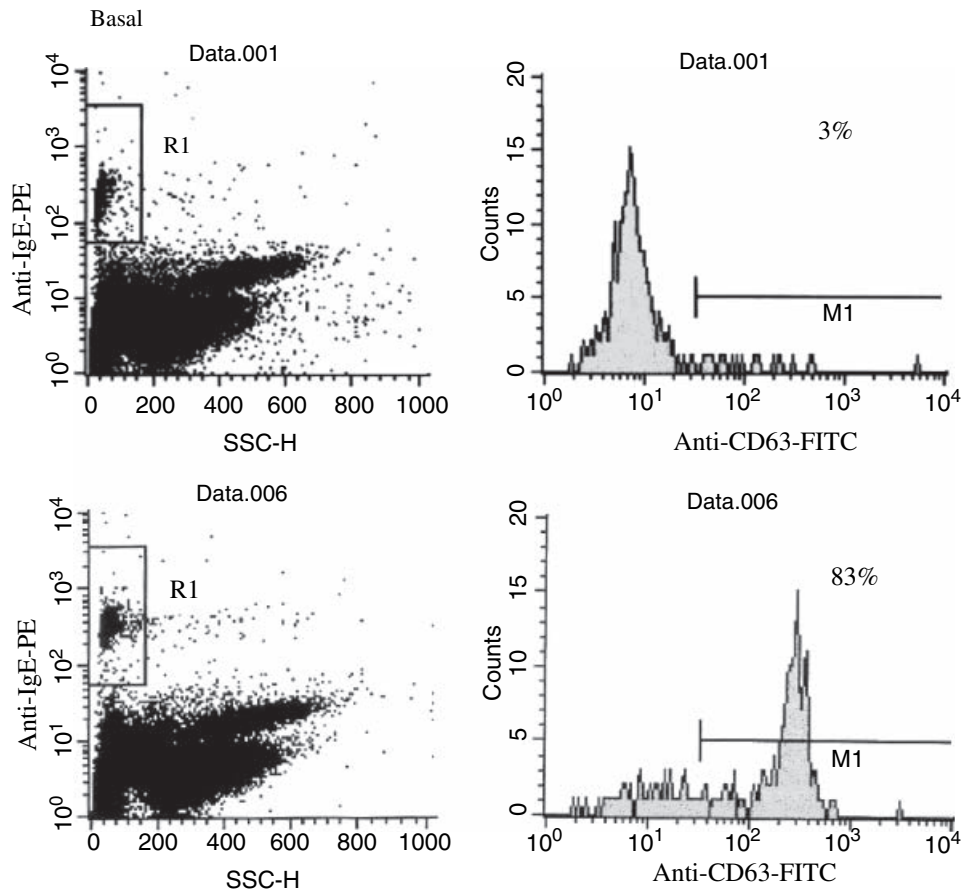


Figure 2. CD63 expression on basophils after incubating with wasp venom in a wasp allergic.

basophils with 0.1 and 1 μg of wasp venom gave the following results with regard to mean basophil activation: 63.39% (SD: 28.99%) and 61.30% (SD: 28.41%). Figure 2 shows basophil activation induced by wasp venom in one of the 50 wasp allergics. In the 20 controls, mean basophil activation after incubating with 0.1 and 1 μg of wasp venom was as follows: 19.38% (SD: 30.29%) and 19.11% (SD: 31.45%). Comparison of percentage of CD63+ basophils shows significant differences between wasp allergics and controls. Applying the Welch test the the corresponding P -value for both wasp allergen concentrations was < 0.0001 . Also for negative and positive controls, percentage of CD63+ basophils did not differ between patients and controls. Data are shown in Fig. 3.

Spearman's correlation coefficients indicated a moderate positive correlation between CAP class and basophil activation in the BAT for both wasp and bee venom. The Spearman's correlation coefficients for 0.1 and 1 μg wasp venom were as follows: $r_{\text{wasp venom 0.1 } \mu\text{g}}$: 0.678, and $r_{\text{wasp venom 1 } \mu\text{g}}$: 0.6453. Spearman's correlation coefficients for 0.1 and 1.0 μg bee venom were as follows: $r_{\text{bee venom 0.1 } \mu\text{g}}$: 0.5956, and $r_{\text{bee venom 1.0 } \mu\text{g}}$: 0.622.

Figure 4 shows the results of ROC analysis for 0.1 and 1.0 μg of wasp allergen. Choosing a cut-off of 15% CD63+ basophils as a positivity threshold yields a

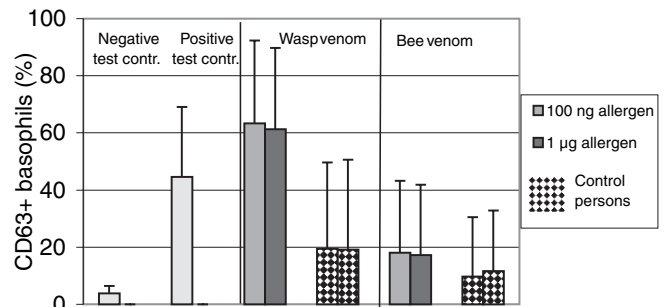


Figure 3. Mean basophil activation (percentage of CD63+ basophils) and standard deviation in 50 wasp allergics and 20 controls after incubation with wasp and bee venom (100 ng and 1 μg).

positive BAT in 46 of 50 patients giving a sensitivity of 92% (95% CI: 81–98%) and a specificity of 80% (95% CI: 56–94%). Reducing the positivity threshold to 10% CD63+ basophils does not increase sensitivity, but reduces specificity to 75%. A positivity threshold of 20% CD63+ basophils would have marginally decreased sensitivity (sensitivity: 90%) without increasing specificity. Therefore, we decided to choose a cut-off of 15% CD63+ basophils for a positive BAT.

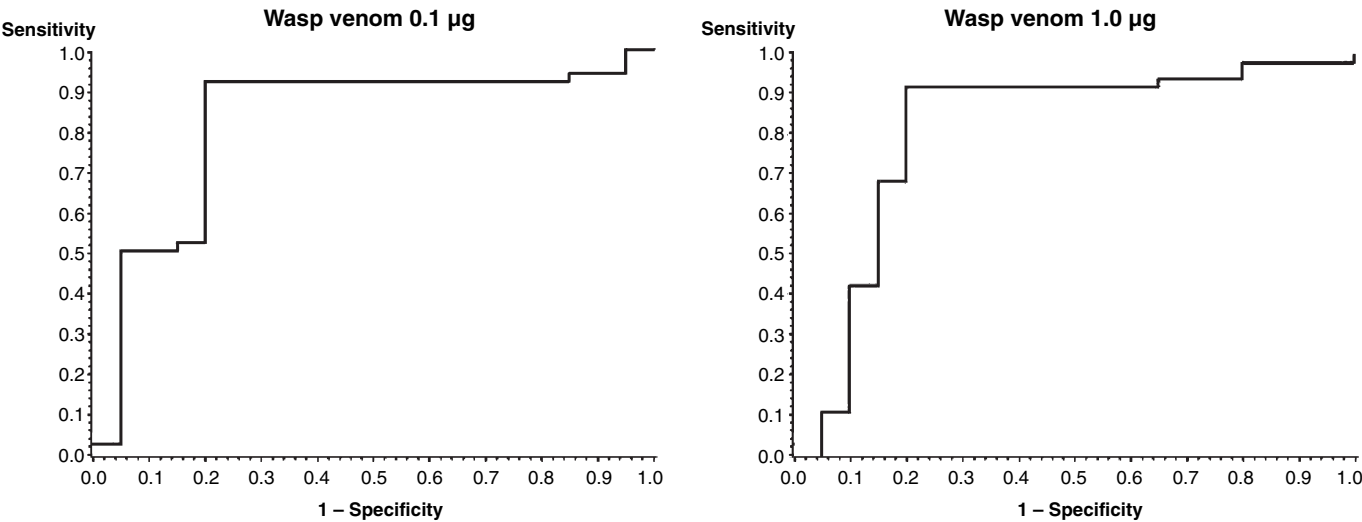


Figure 4. ROC analysis for the CD63 based BAT with 0.1 and 1.0 µg wasp venom. A positivity threshold of 15% CD63+ basophils ('cut-off') yields a sensitivity of 92% and a specificity of 80%. A lower positivity threshold marginally increases sensitivity but considerably reduces specificity.

Three of four controls ($n = 20$) who had a positive BAT reducing specificity also had specific IgE to wasp venom reflecting clinically nonrelevant sensitization. Eleven of 50 wasp allergics and two of 20 controls also had a positive BAT with bee venom, showing double sensitization.

In 25 of 50 wasp allergics who were sting challenged, the BAT was positive before starting VIT. However, in only two subjects the BAT turned negative during VIT. Despite no systemic reaction caused by wasp sting in all patients, the BAT remained positive in 23 of 25 patients,

implying that the BAT is not helpful in predicting the outcome of a sting challenge (Fig. 5). This interpretation was supported by calculation of the Spearman's correlation coefficient which was -0.04231 .

Discussion

In this study, flow cytometry was applied to study allergen-induced basophil activation in wasp venom allergy using a double-staining method with anti-IgE

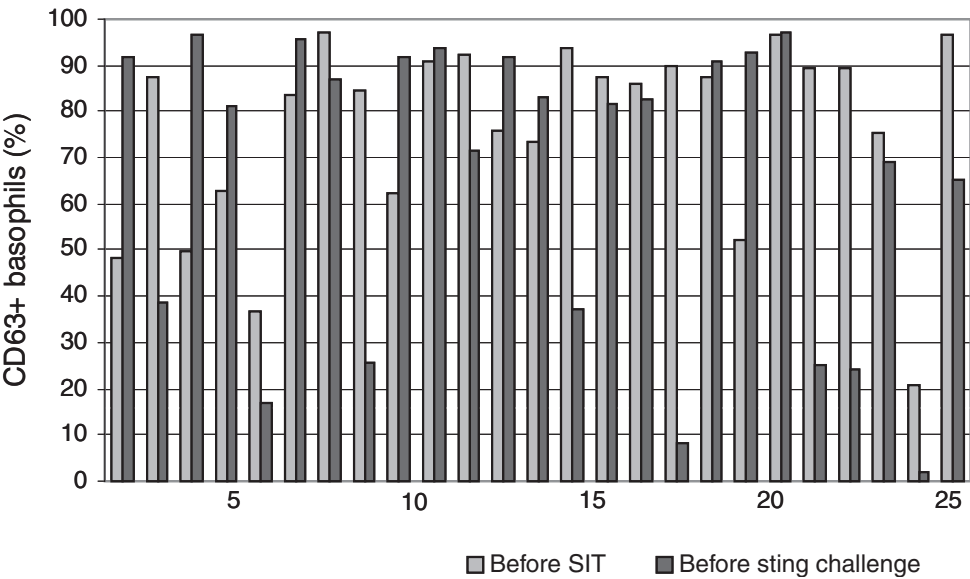


Figure 5. CD 63 expression of basophils after incubation with 0.1 µg wasp venom in 25 wasp allergics before SIT and 6 months after starting SIT prior to sting challenge (cut-off for a positive BAT: 15% CD63+ basophils).

and anti-CD63 mAb as described in the literature (3). Identification of basophils was based on the presence of IgE on the cell surface. The results of this novel technique, the CD63-based BAT, were compared with well-established routine diagnostic methods such as skin testing and allergen-specific IgE. In this study, clinical history was used as a gold standard for evaluation of the diagnostic tests. Diagnostic sting challenges before VIT have been considered to be unethical (1). In addition, the lack of a reaction to a single sting challenge has been shown to be of limited clinical significance, because a subsequent sting can still cause a systemic reaction (18).

Basophil responses are considered to be highly heterogeneous between different donors (19) and comparing different allergens in one donor. Therefore, different allergen concentrations covering several log values have been proposed for basophil-based tests (20). Establishing dose-response curves, we have demonstrated that in wasp and bee allergics induced CD63 expression on basophils by incubating the basophils with wasp or bee venom is not restricted to a single allergen concentration but rather to a range of allergen concentrations which give a high yield of positive results when compared with nonallergic controls (Fig. 1). This is in keeping with the findings for other allergen extracts such as aeroallergens (15) or food allergens (7). However, no general conclusions should be drawn for other allergens or for different allergens such as drugs, as the appropriate allergen concentration remains to be established for each allergen by dose-response curves (21). In this study, pilot experiments revealed individual bell-shaped dose-response curves with limited variation in our subjects. Therefore, in our study, all tests were performed with two allergen concentrations: 0.1 and 1 µg wasp and bee venom. This approach facilitated the evaluation of 'cellular reactivity' of the basophils (i.e. maximal response), but not the 'cellular sensitivity' (i.e. shift of the increasing dose dependent activation) (19). Incubating the basophils with both allergen concentrations yielded the same amount of positive tests for all allergics.

As a positive control, anti-IgE would be most appropriate for allergen-mediated activation of basophils, as the same pathway after crosslinking of FcεRI-receptors on basophils is triggered. However, because of the staining procedure with anti-IgE, another secretagogue has been chosen as a positive control: formyl methionine-containing peptide (fMLP) activates basophils through IgE-independent mechanisms (22). As a consequence, nonreleasing basophils with a defect in signalling IgE-mediated stimuli, occurring in 15–20% of the general population (23), will not be picked up by the fMLP. However, IL-3 co-incubation is able to restore FcεRI-associated protein tyrosine kinase (Syk) expression and IgE-mediated basophil activation (24), decreasing the rate of nonresponders to approximately 5%. As a consequence, positive SPT and detectable IgE concentrations to venom allergens, but a negative outcome using CD63 expression might be explained by this phenomenon,

nonresponding basophils with regard to IgE-mediated stimuli, even if the fMLP control is positive. Mean basophil activation induced by fMLP was lower than the average basophil activation after incubation with the relevant wasp allergen in the wasp allergics, probably because of reduced potency of this secretagogue when compared with the allergens. In 46 of 50 suspected wasp allergics, the CD63-based BAT was positive with a sensitivity of 92%. ROC analysis implied a cut-off of 15% CD63+ basophils for a positive BAT. ROC analysis is a method frequently applied in evaluating and establishing new tests and aims to determine an optimal cut-off for a test to achieve high sensitivity and specificity which is always a compromise (16). The BAT was more sensitive than measurement of allergen-specific IgE reactivity to wasp by the CAP method, which yielded positive results in only 38 of 50 patients giving a sensitivity of only 76%. As the BAT was positive and allergen-specific IgE was negative in 10 patients and allergen-specific IgE was positive yet the BAT was negative in two patients, a combined approach of both *in vitro* tests gives a sensitivity of 96%. Hence, the CD63-based BAT increases the diagnostic yield of *in vitro* tests. This study shows that the CD63-based BAT like other basophil allergy tests has the advantage of an extremely high analytical sensitivity. As selected donors show basophils that respond to antigen with as few as 100–200 molecules of cell-surface IgE (25) basophil-based tests will be positive even if only minute amounts of allergen-specific IgE might not reach the detection limit of the IgE assay (0.35 kU/l). Subsequently, some of the discrepant results (negative IgE, but positive BAT) of the (i.e. nonatopic) donors might be explained by low total IgE levels (< 10 kU/l) and specific IgE below the detection limit of the CAP assay. Interestingly, in our study seven of 10 patients with no proof of allergen-specific IgE had a low level of total IgE.

Intradermal skin testing was positive in all patients demonstrating the high sensitivity of this simple and easy-to-perform basic method of allergy diagnosis (1). Skin testing to confirm sensitization should be performed whenever possible, i.e. no existing contraindications for instance intake of immunosuppressive agents such as glucocorticoids by the patient. Among the 20 controls, there were four subjects who showed positive allergen-specific IgE to wasp or bee venom or a positive BAT or a combination of all demonstrating IgE-mediated sensitization. As selection of the patients was based on clinical history as the 'gold standard', these sensitizations are 'clinically nonrelevant', thus reducing the specificity of the CAP method to 85% and the BAT to 80%.

There was a moderate positive correlation between the degree of basophil activation and IgE reactivity to wasp or bee venom [wasp venom (1 µg): $r = 0.65$; bee venom (1 µg): $r = 0.62$]. These results are in accordance with the results of former studies (7).

Despite great progress in understanding the mechanism of specific immunotherapy and VIT in particular (26, 27),

there is no reliable test which can predict whether a patient on VIT is protected and will have no systemic reaction in case of a future re-sting. Therefore, the potentially life-threatening sting challenge is still recommended (2). Studies monitoring the development of a negative venom skin test response or the fall of venom-specific IgE to low levels were hampered by the low rate of conversion and there have been isolated reports of individuals who had subsequent anaphylaxis after re-stings even after negative venom skin test responses (28).

The use of an *in vitro* cellular test is theoretically justified by the close relationship observed between the *in vitro* measurement of basophil activation and the clinical status (29). Therefore, it was tempting to speculate whether the BAT predicts the outcome of the sting challenge which is in line with other *in vitro* tests. This may be due to the fact that other pathophysiological mechanisms than specific IgE are also determinant in the effector phase of wasp venom allergy (26). Twenty-five of 50 patients consented to a sting challenge which was recommended to all patients 6 months after starting VIT. Although all 25 patients had a positive BAT before starting VIT, there was no decrease of mean basophil activation when the BAT was repeated 6 months later, before the sting challenge. As none of the 25 patients had

a systemic reaction caused by the wasp sting, the BAT is of no benefit in predicting the outcome of the sting challenge which is in line with other *in vitro* tests (30). This may be due to the fact that other pathophysiological mechanisms than specific IgE are also determinant in the effector phase of wasp venom allergy (26, 27). Although it has been speculated that the amount of allergen-induced basophil activation correlates with the clinical status, our results do not support this view. In our controls (negative clinical history) there was proof of allergen-specific wasp venom IgE and positive BAT showing sensitization.

The CD63-based BAT increases sensitivity of *in vitro* diagnosis in wasp venom allergy. Being used in conjunction with measurement of IgE combined *in vitro* diagnosis reaches sensitivity of *in vivo* testing. However, other methods need to be established for routine use to monitor successful VIT and to render sting challenge, feared by most patients, unnecessary.

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