

Basophil Activation Can Predict Clinical Sensitivity in Patients After Venom Immunotherapy

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■ Abstract

Objective: At present, no reliable in vitro test is available to monitor the success of specific venom immunotherapy (VIT) in preventing insect venom anaphylaxis. We investigated usefulness of the basophil activation test (BAT) in predicting the outcome of sting challenge in bee venom-allergic patients after VIT.

Patients and Methods: Twenty-one patients with bee venom anaphylaxis at the end of VIT and 6 control participants were enrolled. BAT (flow-cytometric evaluation of allergen-induced expression of CD63), skin testing, and specific immunoglobulin (Ig) E determination were performed prior to sting challenge.

Results: Five of the 21 patients (23.8%) reacted to sting challenge. At a bee venom concentration of 100 ng/mL, the mean proportion of basophils expressing CD63 was 56% in reactors and 13.2% in nonreactors ($P=.0321$). Four of the 5 reactors had positive results and 14 of the 16 nonreactors had negative results. Thus, using 18.4% and 21.6% (receiver operating characteristic curve analysis) as the cutoff for expression of the CD63 marker, the positive and the negative predictive values were 67% and 93%, respectively, and specificity and sensitivity for BAT were 80% and 83%, respectively. However, at a concentration of 1000 ng/mL, no significant differences in basophil activation were observed between reactors and nonreactors.

Conclusion: We found BAT to be a helpful tool in predicting the clinical sensitivity of bee venom-allergic patients after VIT (correlation between BAT at submaximal venom concentration and sting challenge).

Key words: Basophil activation test. CD63. Bee venom anaphylaxis. Sting challenge. Specific venom immunotherapy.

■ Resumen

Objetivo: Hasta la fecha, no existe ninguna prueba in vitro fiable que permita evaluar el éxito de la inmunoterapia específica con veneno para prevenir la anafilaxia por veneno de insectos. En este estudio se investigó la utilidad del test de activación de basófilos (TAB) en la predicción del resultado de la prueba de provocación con picadura en pacientes alérgicos al veneno de abeja tras la inmunoterapia con veneno.

Pacientes y métodos: Se incluyeron 21 pacientes con anafilaxia por veneno de abeja al final de la inmunoterapia con veneno y 6 participantes control. Se llevó a cabo un TAB (citometría de flujo de la expresión de CD63 inducida por alérgenos), una prueba cutánea y la determinación de IgE específica antes de la prueba de provocación con picadura.

Resultados: Cinco de los 21 pacientes (23,8%) reaccionaron a la prueba de provocación con picadura. A una concentración de veneno de abeja de 100 ng/ml, la proporción media de basófilos CD63 positivos fue del 56% en los pacientes con reacción y del 13,2% en los pacientes sin reacción ($p= 0,0321$). Cuatro de los 5 pacientes con reacción dieron positivo y 14 de los 16 pacientes sin reacción dieron negativo en TAB. Así, tomando el 18,4% y el 21,6% de basófilos CD63 positivos como valores de corte, los valores predictivos positivos y negativos fueron del 67% y el 93%, respectivamente, y la especificidad y la sensibilidad de TAB fueron del 80% y el 83%, respectivamente. Sin embargo, a una concentración de 1.000 ng/ml, no se observaron diferencias significativas en la activación de basófilos entre el grupo con reacción y el grupo sin reacción.

Conclusión: El test de activación de basófilos es una herramienta útil para predecir la sensibilidad clínica de los pacientes alérgicos al veneno de abeja tras la inmunoterapia con veneno (correlación entre el TAB a una concentración submáxima de veneno y las pruebas de provocación con picadura).

Palabras clave: Test de activación de basófilos. CD63. Anafilaxia por veneno de abeja. Prueba de provocación con picadura. Inmunoterapia específica con veneno.

Introduction

Systemic allergic reactions (SARs) to Hymenoptera venom are estimated to occur in about 0.3-7.5% of the general population [1]. Specific venom immunotherapy (VIT) is highly effective in preventing insect venom anaphylaxis [2-4], although not all patients are protected during re-sting after completing VIT, and questions on duration and discontinuation of treatment, as well as the reasons for its failure, remain unanswered [5-6].

According to current European Academy of Allergy and Clinical Immunology (EAACI) guidelines [6], the potential criteria for stopping VIT include duration of treatment (3-5 years), loss of sensitization, absence of severe SARs (to VIT injections, field stings, or both), and younger age. However, venom-specific serum immunoglobulin (Ig) E is detectable in more than 75% of cases [7], and skin tests give negative results in about 25% of patients, even after a 5-year VIT schedule [8]. In addition, neither skin testing nor specific IgE determination has proven to have a predictive value for the outcome of re-sting after completion of VIT [9-11]. Specific IgG antibodies have no predictive role with regard to re-sting risk after stopping VIT, and, although a correlation between elevated serum levels of specific IgG and better tolerance of VIT or increased serum tryptase concentration and reduced VIT efficacy has been reported [12,13], no reliable *in vitro* marker for evaluation of venom tolerance exists.

Thus, there is still an urgent need to establish an *in vitro* method that can evaluate the success of VIT and that can act as an alternative to the rather problematic sting challenge with the relevant living insect (difficult-to-perform, risk of SARs). This could help not only to differentiate whether patients require long-term high-dose treatment or they have already achieved immunologic tolerance, but also to monitor the duration of the protective effect of VIT.

The basophil activation test (BAT) is based on flow cytometric quantification of allergen-induced activation markers on the basophil membrane [14]. Activation of basophils can be evaluated by assessing the expression of several membrane molecules (eg, CD63, CD123, and CD203c). CD63, a member of the transmembrane-4 superfamily, is rapidly mobilized on the basophil membrane by different degranulation stimuli, and is probably the most widely used marker in basophil-based assays. Highly specific and sensitive BAT has been proposed as a complement to the diagnostic procedure (especially when there is a discrepancy between the results for skin testing and specific IgE determination) in allergy to food [15], pollen [16], dust mite [17], latex [18], drug [19], and Hymenoptera venom [20]. However, current data on the usefulness of BAT in monitoring VIT efficacy are controversial.

The aim of our study was to investigate whether basophil sensitivity, as evaluated by the BAT, correlates with clinical reactivity (sting challenge) in bee venom-allergic patients after VIT and whether it might help to identify venom-tolerant patients. We expected significantly higher basophil CD63 expression in patients who continue to react to sting challenge than in those who do not.

Material and Methods

Patients

Twenty-one patients with bee venom-induced SAR (8 women and 13 men, mean [SD] age 46.1 [14.34] years) were selected for the study on the basis of their clinical history (grade II-IV SAR classified according to the H. Mueller schedule), positive skin test results, and/or elevated specific IgE levels. These otherwise healthy patients were treated with purified aqueous bee venom extract in accordance with EAACI standards [6]. The mean duration of VIT (maintenance phase) was 4.4 years (range, 3.1-5.2 years). BAT, repeated venom skin testing, and specific IgE determination were performed at the end of VIT before sting challenge. Serum levels of bee venom-specific IgE were measured using the CAP-FEIA system (Phadia, Uppsala, Sweden) according to the manufacturer's protocol. Skin tests were carried out with purified bee venom extract (ALK-Abelló, Hørsholm, Denmark) as recommended by EAACI [21]. The mean time between basophil testing and sting challenge was 5 weeks. Six healthy nonsensitized individuals (4 women and 2 men; mean [SD] age, 38.3 [7.6] years) with no history of allergy to bee venom were enrolled as controls. To analyze the relationship between the venom allergen concentration and basophil response and to identify the appropriate stimulation concentrations, we tested blood specimens from 4 selected patients with systemic symptoms of venom allergy before immunotherapy. All participants signed the informed consent and the project was approved by the local Institutional Review Board.

Basophil Activation Test

The BAT was performed according to the modified manufacturer's recommendations (Basotest, Orpegen Pharma, Heidelberg, Germany). Briefly, 100 μ L of heparinized blood was incubated with 20 μ L of stimulation buffer (containing interleukin 3) for 10 minutes at 37°C. Next, allergen (standardized bee venom extract at the final concentration of 100 and 1000 ng/mL [ALK-Abelló]), the chemotactic peptide N-formyl-met-leu-phe, and anti-IgE antibody (at a final concentration of 10 μ g/mL, [Immunotech, Marseille, France]) as a positive control, or washing solution as a negative control were added (100 μ L, incubation 20 min at 37°C). Basophil degranulation was stopped by chilling on ice for 5 minutes. Subsequently, the cells were stained with anti-IgE/phycoerythrin and anti-CD63/fluorescein isothiocyanate monoclonal antibodies. After erythrocyte lysing and washing, basophil activation was evaluated using the Cytoron Absolute flow cytometer and Immunocount software (both from Ortho Diagnostics System, Raritan, New Jersey, USA). Basophils were gated as IgE-positive cells and further analyzed for CD63 expression. The minimal number of acquired basophils per sample was 1000. Results are expressed as the mean (SD) percentage of CD63⁺ basophils.

Sting Challenge

Only patients with no symptoms of acute disease, normal findings on physical examination, and normal pulmonary

function underwent sting challenge. Emergency equipment was available during the procedure, an intravenous catheter was inserted, and blood pressure and ECG were monitored. Sting challenge was performed as recommended in the EAACI Position Paper [22]. Individual live honeybees were kept in place for at least 60 seconds and the reaction was classified (grade 0-IV). We recently reviewed the indication for and methodology of sting challenge [23,24].

Statistical Analysis

The distribution of data was nonnormal. The nonparametric Mann-Whitney test was used to compare samples; the χ^2 and Fisher exact tests were used to compare proportions. *P* values $\leq .05$ were considered significant. The quality of the BAT was expressed in terms of sensitivity and specificity. The predictive values and likelihood ratios enabling the effect of the test on the probability of an outcome to be quantified were applied using a simplified form of the Bayes theorem. Analyses were performed with StatsDirect Statistical Software (StatsDirect, Altrincham, UK).

10 000 ng/mL elicited significantly lower expression. Similarly, 2 receiver operating characteristic (ROC) curves showed that concentrations of 100 and 1000 ng/mL can optimally discriminate between reactive patients and negative controls (threshold of 18.4% for 100 ng/mL and 21.6% for 1000 ng/mL; Figure 2a, 2b).

At a concentration of 100 ng/mL, the mean proportion of CD63⁺ basophils was 56.24% (35.15%) in reactors, compared to 13.21% (18.95%) in nonreactors (*P*=.0321). Figure 3 shows the median and quartiles of CD63 expression in reacting and nonreacting patients. As 4 of 5 reactors had positive BAT results and 14 of 16 nonreactors had negative BAT results (*P*=.0116), the positive predictive value for the test was 67% and the negative predictive value 93%. The likelihood ratio for a positive test result and a negative test result was 6.4 (95% confidence interval [CI], 1.76-23.89) and 0.2286 (95% CI, 0.0411-0.744), respectively.

A bee venom concentration of 1000 ng/mL did not reveal significant differences in basophil activation between reactors and nonreactors (72.10% [40.65%] vs 36.50% [31.64%] CD63⁺

Table 1. Demographic and Clinical Data of Bee Venom-Allergic Patients^a

	Sex		Mean Age, y	Reaction Grade	Duration of VIT, y	Specific IgE, kU _A /L
	Male	Female				
All patients N = 21	13	8	46.14 (14.34)	3.31	4.4 (1.22)	4.38 (4.83)
Reactors n=5	2	3	55.6 (15.31)	3.4	5.0 (1.73)	9.14 (6.99)
Nonreactors n=16	11	5	43.119 (13.14)	3.3	4.22 (1.02)	2.89 (2.86)
Statistical significance	<i>P</i> =.325		<i>P</i> =.037	<i>P</i> =.855	<i>P</i> =.152	<i>P</i> =.091

Abbreviations: Ig, immunoglobulin; VIT, venom immunotherapy.

^a Data expressed as mean (SD), unless otherwise indicated.

Results

Re-exposure to Bee Sting

Five of the 21 patients (23.8%) reacted to re-sting. In 4, the reactions were mild cutaneous, and 1 patient reported a drop in blood pressure with no need for aggressive treatment. Reactors were significantly older than nonreactors (mean age 55.6 vs 43.2 years, *P*=.037). However, no significant differences were observed for sex, severity of previous SARs (reaction grade), level of sensitization, or VIT duration between the groups (Table 1).

Basophil Activation Test

The first dose-response experiments are summarized and shown in Figure 1. The percentage of CD63⁺ basophils stimulated by venom in the controls ranged from 4% to 13%. In contrast, systemic reactors showed dose-related up-regulation of basophil activation with the maximal response at 1 000 ng/mL; concentrations of 1 to 100 and

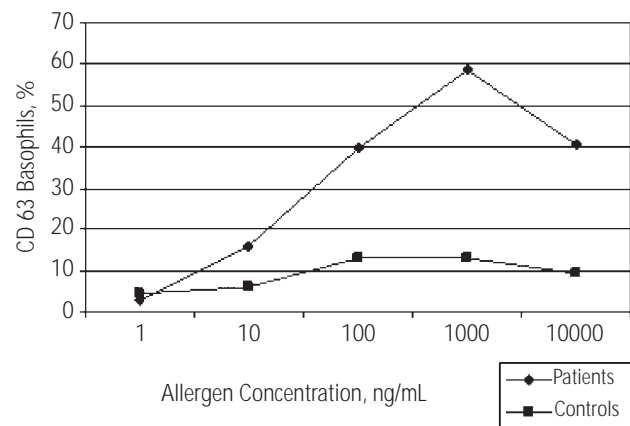


Figure 1. Dose-response curves of CD63 expression related to bee venom allergen concentration (1 ng/mL up to 10 000 ng/mL) in sensitized individuals and healthy controls.

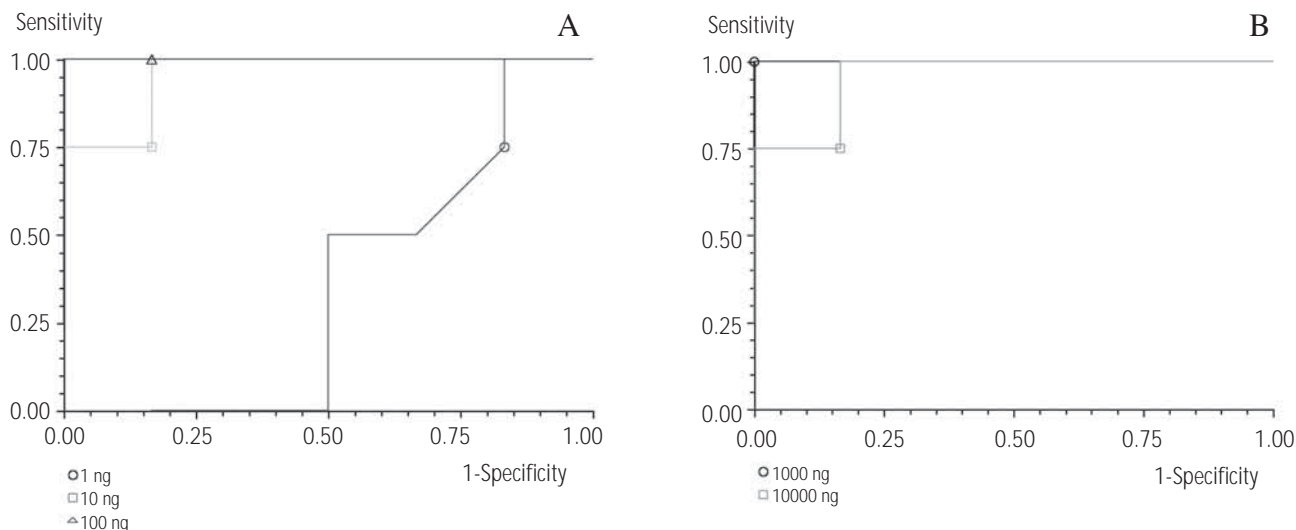


Figure 2. ROC plot curves of venom allergen induced expression of the marker CD63 in venom-allergic patients (n=4) and healthy controls (n=6), allergen concentration: A, 1 to 100 ng/mL. B, 1000 to 10 000 ng/mL.

basophils; $P=.0616$). Eighty percent of reactors had positive results and 43.7% of nonreactors had negative results ($P=.408$). Thus, the positive and negative predictive values for BAT at this higher bee venom concentration were 31% and 88%, respectively. The proportion of positive and negative BAT results (CD63 expression) in reactors and nonreactors at both concentrations is shown in Table 2. Patients who tolerated a sting challenge after VIT tended to have a lower specific IgE response to venom allergen than those who reacted, although the difference was not statistically significant (mean serum IgE level 8.80 [6.39] kU_A/L vs 16.25 kU_A/L ; $P=.398$).

In the controls, mean (SD) CD63 expression at 100 and 1000 ng/mL was 13.3% (4.77%) and 13.1% (4.57%), respectively. The basophils of all participants exhibited a clear positive response to positive stimulation controls.

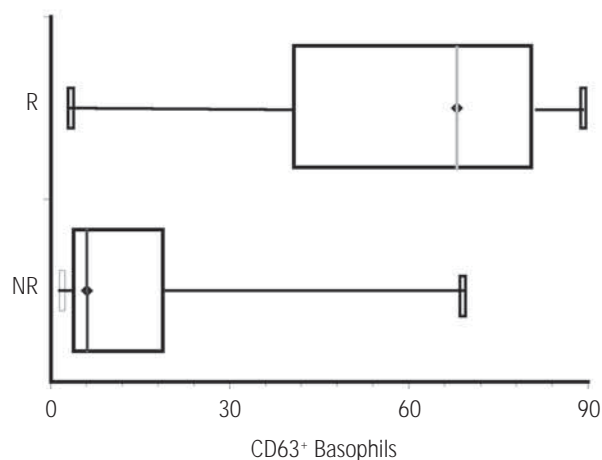


Figure 3. Median and quartiles of CD63 expression on basophils at bee venom concentration of 100 ng/mL. R indicates reactors; NR, nonreactors.

Table 2. Proportion of Positive and Negative BAT Results (Expression of CD63) in Patients Reacting or Not Reacting to Sting Challenge (Basophil Stimulation at the Concentration 100 and 1000 ng/mL).

	Reactors, No. (%)	Nonreactors, No. (%)	Statistical Significance
CD63-positive 100 ng/mL	4/5 (80)	2/16 (12.5)	$P=.0116$
CD63-negative	1/5 (20)	14/16 (87.5)	
CD63-positive 1000 ng/mL	4/5 (80)	9/16 (65.3)	$P=.408$
CD63-negative	1/5 (20)	7/16 (43.7)	

Abbreviations: BAT, basophil activation test.

Discussion

Despite considerable progress in understanding the mechanism of allergen-specific VIT [25], there are no reliable in vitro assays that can predict whether a patient on VIT is protected from SAR in the case of a re-sting. Therefore, sting challenge remains the only method that can evaluate the success of VIT [22]. However, several practical and ethical issues (risk of SARs) limit its application [23,24]. As commonly used diagnostic tools for venom allergy (skin testing, specific IgE, IgG4 determination) do not seem to be suitable for VIT monitoring [9-11], attention has recently turned to in vitro cellular tests such as the sulfidoleukotriene release assay and the BAT. In vitro basophil stimulation resembles the pathophysiology of anaphylactic reaction in vivo; therefore, we and other authors believe that a decreased basophil response (suppressed expression of the marker CD63 on the cell membrane) during or after VIT may correlate with in vivo tolerance of culprit venom in allergic patients. To date, 3 authors have addressed this topic, and reported data are not fully consistent [26,27,29].

In this study, we demonstrated that BAT in bee venom-allergic patients treated by VIT for at least 3.1 years correlated well with sting challenge (80% of reactors showed positive BAT results and 87.5% of nonreactors showed negative BAT results). Therefore, CD63 expression on the basophils of patients who continue to react to sting challenge was significantly higher than on those of nonreacting patients. This was only the case, however, for the bee venom concentration of 100 ng/mL. We did not find any statistically significant difference in basophil CD63 expression at 1000 ng/mL when comparing reactors with nonreactors (although 80% of reactors had positive BAT results, only 43.7% of nonreactors had negative results). Ebo et al [26] reported no significant changes in CD63 expression after 5 days of semirush VIT using 3 allergen concentrations (wasp venom at 0.01, 0.1, and 10 µg/mL). In contrast, the percentage of basophils expressing CD63 at a submaximal venom concentration of 0.01 µg/mL and also at 0.1 µg/mL was significantly lower in wasp-allergic patients who underwent a 3-year maintenance VIT than the pretreatment values of the systemic reactors. This was not demonstrable at 10 µg/mL. In agreement with this finding, submaximal basophil stimulation with wasp venom (0.01 µg/mL) in other patient groups led to a significant decrease in CD63⁺ expression after 6 months of VIT when compared to pretreatment BAT values, whereas at higher allergen concentrations the basophil response was not influenced by VIT. As a sting challenge was not performed in that study, a direct comparison cannot be drawn, and although 26.7% of the patients treated using a 3-year VIT schedule experienced a field sting without SARs, some of them had a negative BAT result at only 0.01 and 0.1 µg/mL, and some remained positive.

On the contrary, Erdmann et al [27] observed neither a correlation between sting challenge and BAT nor a significant decrease in mean basophil activation when BAT values were compared before and 6 months after starting VIT. Ebo et al [26] suggested that this might be caused by the fact that the time-course of BAT during VIT was assessed with a stimulation concentration resulting in maximal cell response.

In another study, Ebo et al [28] clearly demonstrated that long-term VIT is able to decrease basophil sensitivity, although remarkable differences in cell response were observed after at least 3 years of treatment, and a strong decrease in basophil sensitivity can be found even after 5 years of VIT or 1 year after stopping VIT. The failure to predict the result of sting challenge using BAT in the Erdmann study [27] can be also explained by the short period of immunotherapy (6 months), which might reflect possible different pathophysiological mechanisms of systemic degranulation during anaphylaxis and fusion of the intracellular granules with the basophil cell membrane.

Similarly, Gober et al [29] demonstrated changes in basophil activation during VIT and suggested that the venom concentration of 1000 ng/mL was too high for basophil stimulation, compared with the hypothetical estimate of venom concentration in blood after a sting. Comparison of the results of this study is limited due to the fact that 14 out of 21 basophil donors experienced large local reactions. The basophil response is considered to be highly heterogenous, not only in different donors, but also when different allergens are compared; the clinical course of patients with systemic reactions to bee and wasp venom is also different. Therefore, direct comparison of our results for bee venom-sensitized patients and the results of Ebo [28] and Erdmann [27] is difficult, as their studies addressed patients with wasp venom allergy only.

The cross-sectional study from Peternelj et al [30] comparing patients after VIT reported the lower reactivity of basophils of patients who tolerated field stings at a venom concentration of 100 ng/mL only. A submaximal venom concentration of 100 ng/mL was identified as optimal for discrimination between reactors and nonreactors, as BAT results at higher or lower venom concentrations (1000 and 10 ng/mL) were comparable in these 2 groups of patients. However, the design of our study does not allow us to directly compare the results of field-stung and intentionally challenged patients. Those of our findings that are consistent with the study by Peternelj et al indicate the importance of changes in basophil sensitivity (stimulation with a submaximal concentration), and not reactivity (maximal venom concentration), when evaluating the success of VIT. Finally, Brown et al [31] investigated several assays (BAT, mediator release tests, venom-induced leukocyte proliferation, cytokine production) to follow up VIT in ant-allergic patients (*Myrmecia pilosula*), although none proved useful. We also found that reactors were significantly older than nonreactors. This supports the observation that older patients usually require longer VIT [6].

To our knowledge, this is the first report of such a close correlation between the outcome of sting challenge and BAT in bee venom-allergic patients treated by VIT. We demonstrated that BAT at allergen concentrations lower than those giving a maximal cellular response was able to differentiate between individuals still reacting to sting challenge after VIT. In particular, the predictive value of negative results was very high.

Our study is limited by its cross-sectional design. We did not compare basophil sensitivity before and after VIT, but used clinically comparable groups of patients from the same treatment period. In addition, we must remember that a single negative sting challenge does not definitely confirm the absence of anaphylaxis [32].

In summary, higher basophil sensitivity to bee venom allergens is associated with a potentially reduced effect of VIT and persistent clinical reactivity. Therefore, the BAT can be a viable tool for predicting the outcome of immunotherapy. Nevertheless, further studies with a larger numbers of venom-allergic patients treated by VIT are needed.

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