

Diagnosis of venom allergy by flow cytometry. Correlation with clinical history, skin tests, specific IgE, histamine and leukotriene C4 release

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Abstract

Background Potent allergens such as hymenoptera venoms are capable of inducing severe and life threatening clinical reactions. Percentage of false negative results obtained by the usual diagnostical methods is comprised between 10 and 25%

Objective Evaluation of the sensitivity and the specificity of cellular tests and particularly evaluation of a new flow cytometric method.

Methods Forty-five allergic patients having experienced a local, a systemic reaction or an anaphylactic shock and 10 controls having undergone hymenoptera stings without clinical reactions were selected on the basis of the clinical history, skin tests and specific IgE. Three cellular tests were performed on the same cell suspensions and in the presence of 2 ng/mL of rIL3: histamine release (RIA), leukotriene C4 release (ELISA) and basophil activation test (flow cytometry after double anti-IgE FITC, anti-CD63 PE labelling).

Results As compared to the clinical history, sensitivities of skin tests, specific IgE, flow cytometry, histamine release and leukotriene release were, respectively; 85%, 88%, 100%, 89% and 100%. Flow cytometric analysis of basophil activation showed a significant decrease of the mean fluorescence density and number of IgE positive cells and a significant increase of the number of CD63 positive cells. The 10 controls tested by flow cytometry were negative.

Conclusion As compared to the clinical history and to the other parameters tested here, flow cytometry showed a high sensitivity and a high specificity. The excellent correlation observed between this method and the other cellular tests such as histamine and leukotriene release are in favour of the specificity of flow cytometry and in favour of the use of this method for venom allergy diagnosis.

Keywords: Venom allergy, allergy diagnosis, histamine release, leukotriene release, flow cytometry, CD63.

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Introduction

Clinical reactions induced by hymenoptera venoms may be very severe and even life threatening. (0.09–0.45 deaths per million within the general population). More frequently the clinical reaction observed after an hymenoptera sting is

local or loco-regional (2 to 19% of the general population) or systemic (0.8–3.9% of the general population) [1,2], these frequencies being certainly underestimated.

Venom allergy diagnosis is based on clinical history, skin tests, specific IgE and, less frequently, on histamine release [3]. 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 [4].

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Moreover, recent results obtained by cellular tests such as histamine release [3], LTC₄ release [3,5–8] and flow cytometry [7,9–11] are in favour of the greater specificity of the measurement of allergen induced basophil activation than circulating IgE determination.

The flow cytometric method described here is based on the double anti-IgE FITC, anti-CD63 PE labelling of activated basophils. The CD63 marker is a 53-kDa glycoprotein present on the lysosome membrane of various cell types and is expressed with a high density on activated basophil membrane [12–14]. It may be considered as a real degranulation marker.

Recent results obtained by our group [11] have shown that the vast majority of the IgE positive cells selected by the cytometer express the immuno-phenotype of human basophils. A variable fraction of activated basophils may bear aggregated platelets, but the expression of the CD63 marker on basophil membrane, due to the fusion of the intracellular granules with the cytoplasmic membrane, is the result of basophil and not platelet activation [11].

Here we present results obtained by histamine release, leukotriene C₄ release and basophil activation (CD63 μ p regulation on activated basophil membrane) and their correlation with clinical history, skin tests and specific IgE.

Materials and methods

Selection of patients

Forty-five patients (11–57 years old, mean 45) having experienced a local reaction ($n = 12$), a systemic reaction (Quincke oedema, generalized urticaria, $n = 25$) and an anaphylactic shock ($n = 9$) have been selected on the basis of clinical history, skin tests, specific IgE and histamine release. The identified insect was a wasp ($n = 35$), a bee ($n = 8$) and a hornet ($n = 2$). Sixteen control subjects (28–47 years old, mean 39) having been stung without reaction were selected in parallel. Skin tests were not performed on controls for ethical reasons.

Skin tests

Skin tests were performed by intradermal reaction (IDR) (Dome Hollister Stier extracts) in parallel with a negative (diluent) and a positive (histamine) control. The positivity threshold was established at 4 mm of weal diameter.

Specific IgE determination

The specific IgE determination in serum was performed by the CAP-RIA method (Pharmacia, Sweden) according to the manufacturer's recommendations.

Allergen-induced basophil activation

Whole blood taken on EDTA was centrifuged (500g, 10 min, 4 °C), the buffy coat was pipetted out (400 μ L per 10 mL of whole blood) and resuspended in an hepes-EDTA buffer (hepes 20 mM, NaCl 130 mM, KCl 5 mM, EDTA Na₃ 0.5 mM pH 7.4).

For the experiments performed after recombinant interleukin-3 (IL-3) priming, the washed leucocyte suspension was mixed v/v with a 2-ng/mL solution of rIL3 diluted in an hepes-calcium buffer and incubated 15 min at 37 °C. Thirty μ L of the cell suspension were distributed in microtitre plate wells and mixed in parallel with 30 μ L of hepes-calcium buffer (hepes 20 mM, NaCl 130 mM, KCl 5 mM, Ca++ 2 mM, Mg++ 1 mM, pH 7.4) (negative control), 30 μ L of anti-IgE diluted to 10–3 in the same buffer (positive control) and 30 μ L of 3 dilutions of the tested allergen extract diluted in the same buffer to 1 μ g/mL, 0.20 μ g/mL and 0.04 μ g/mL. Plates were incubated 45 min at 37 °C and 150 μ L of hepes-EDTA buffer were added in every well. Plates were centrifuged (500g, 10 min, 4 °C). Supernatants were either used immediately for histamine or leukotriene C₄ (LTC₄) determination or stored at –20 °C in polycarbonate microtitre plates if LTC₄ determination was to be delayed in order to avoid LTC₄ adsorption on polystyrene solid phase. Cell pellets were used for the flow cytometric analysis of basophil activation. An anti-IgE positive control (Polyclonal goat anti-IgE, ATAB, USA, 0.2 μ g/mL) was run in parallel.

LTC₄ Release test (LRT)

The amount of LTC₄ released in the supernatants was measured by ELISA (Stallergenes, France) using a monoclonal antibody showing a negligible cross reaction, according to the manufacturer, with leukotriene D₄ (LTD₄) and leukotriene E₄ (LTE₄). The sensitivity of the test was 50 pg/mL.

Histamine release test (HRT)

The amount of histamine released within the incubation medium was measured by RIA (Immunotech, France), results being expressed in percentage release (spontaneous release (<5%) deduced) vs the total histamine content of the tested leucocyte suspension performed by adding 150 μ L of distilled water to an aliquot of the cell suspension followed by two steps of freezing and thawing.

Basophil activation test (BAT)

After the allergen stimulation step and removal of 150 μ L of the supernatants, 40 μ L of hepes-EDTA were added to the cell pellets and plates were centrifuged (500g, 5 min, 4 °C). Forty μ L of supernatants were discarded and 20 μ L of cell

suspensions were mixed with the two antibodies (anti-IgE fluorescein isothiocyanate (FITC) and anti-CD63 phycoerythrin (PE)). After 20 min at room temperature in the dark, 100 µL of lysing solution (Ortho diagnostics, USA) were added in the wells in order to lyse the remaining erythrocytes. After exactly 3 min at room temperature, 100 µL of hepes-EDTA were added and plates were centrifuged (500g, 5 min). Leukocytes were resuspended in 250 µL of hepes-EDTA and analysed by flow cytometry using a bi-colour flow cytometer (Cytoron absolute, Ortho diagnostics, USA). Basophils were gated within the lymphocyte region of the small (FSC) and large angle (RT-SC) scatter pattern. The anti-IgE FITC brightly fluorescent population (comprising a vast majority of basophils) was gated and anti-CD63 positive and negative cells were separated by an electronic threshold. 150–500 basophils were systematically counted. Results were expressed in percentage activation calculated according to the formula:

Percentage activation = Number of anti-IgE FITC + CD63 PE+ cells/number of anti-IgE FITC+ cells

Other parameters were given simultaneously by the cytometer such as the percentage of the IgE-positive population and the related mean fluorescence density.

Reagents

The immunological reagents (anti-IgE FITC (Caltag, USA), anti-CD63 PE (Caltag, USA), human rIL3 (Peprotech, USA) were purchased from Tebu (France). All the other reagents were purchased from Sigma (France)

Statistical analysis

According to our experience and to the results obtained here by the three cellular tests (BAT, HRL and LRT) for the allergen-free controls, the respective positivity thresholds used here were 5%, 6% and 160 pg/mL for, at least, two successive allergen dilutions. The absolute number of basophils contained in the leucocyte suspensions was not counted, the interpretation of the tests depending on the number of basophils counted by the cytometer for BAT (more than 100), the total histamine content for HRL (more than 10 ng/mL) and the amount of LTC4 released for the anti-IgE controls (more than 300 pg/mL)

The statistical significance of the results obtained by these methods was performed by the distribution free Wilcoxon and Spearman tests.

Results

Variation of the percentage of IgE+ cells and variation of the related mean intensity of fluorescence.

Twelve patients allergic to vespula venom and 12 controls were selected. We compared the percentage IgE+ cells

and the related mean intensity of fluorescence (MIF) calculated by the cytometer for the two populations of subjects.

For the control subjects (Fig. 1) no statistical difference was observed between the allergen free controls and the three allergen dilutions and for both parameters. For the allergic patients (Fig. 1) a significant decrease of the number of IgE+ cells ($P < 0.0001$) in parallel with a significant decrease of the MIF ($P < 0.01$) was observed between the allergen free controls and the three allergen dilutions. For the two populations, a significant decrease of both parameters was also observed for the anti-IgE controls (positive controls). The comparison of the mean values related to the allergen free controls showed a higher percentage IgE+ cells and a lower MIF for the control subjects than for the allergic patients, but these differences were not significant.

Comparison of the sensitivities of the different biological parameters expressed vs. the clinical history.

The sensitivities of the different biological parameters defined as the percentage of positive tests obtained for the allergic patients population were 85% for skin tests, 88% for specific IgE, 100% for BAT, 89% for HRL in the presence of IL3 (79% in the absence of IL3) and 100% for LRT.

Specificity of BAT vs the clinical history was 100% as all control subjects were negative

Correlation of BAT with specific IgE, HRL and LRT.

Correlation between BAT and the three other parameters (Table 1) was significant for HRL ($P < 0.0001$) and LRT ($P = 0.038$) and not significant for specific (IgE)

Twenty-five HRL were performed in the presence of IL3 and 13 in the absence of IL3. For for the 1 µg/mL allergen concentration the percentage histamine released was inferior to the percentage of CD63 positive cells ($P < 0.0003$) both in the presence (TAB 56.8% ± 24, HRL 38.9% ± 23) or in the absence (TAB 54.4% ± 27, HRL 14.3% ± 15) of IL3 for HRL. HRL results obtained in the presence and in the absence of IL3 being also significantly different ($P < 0.001$).

Results obtained by each cellular test (Table 2) for the three types of clinical reaction were not significantly different.

Conclusion

Results presented here demonstrate that basophil activation

Table 1. Correlation of BAT with specific IgE (sIgE), HRL and LRT. (Spearman distribution free test, NS = not significant)

	Nb of data	<i>r</i>	<i>P</i>
BAT/sIgE	45	0.23	NS
BAT/HRL	39	0.61	< 10 ⁻⁴
BAT/LRT	31	0.38	0.038

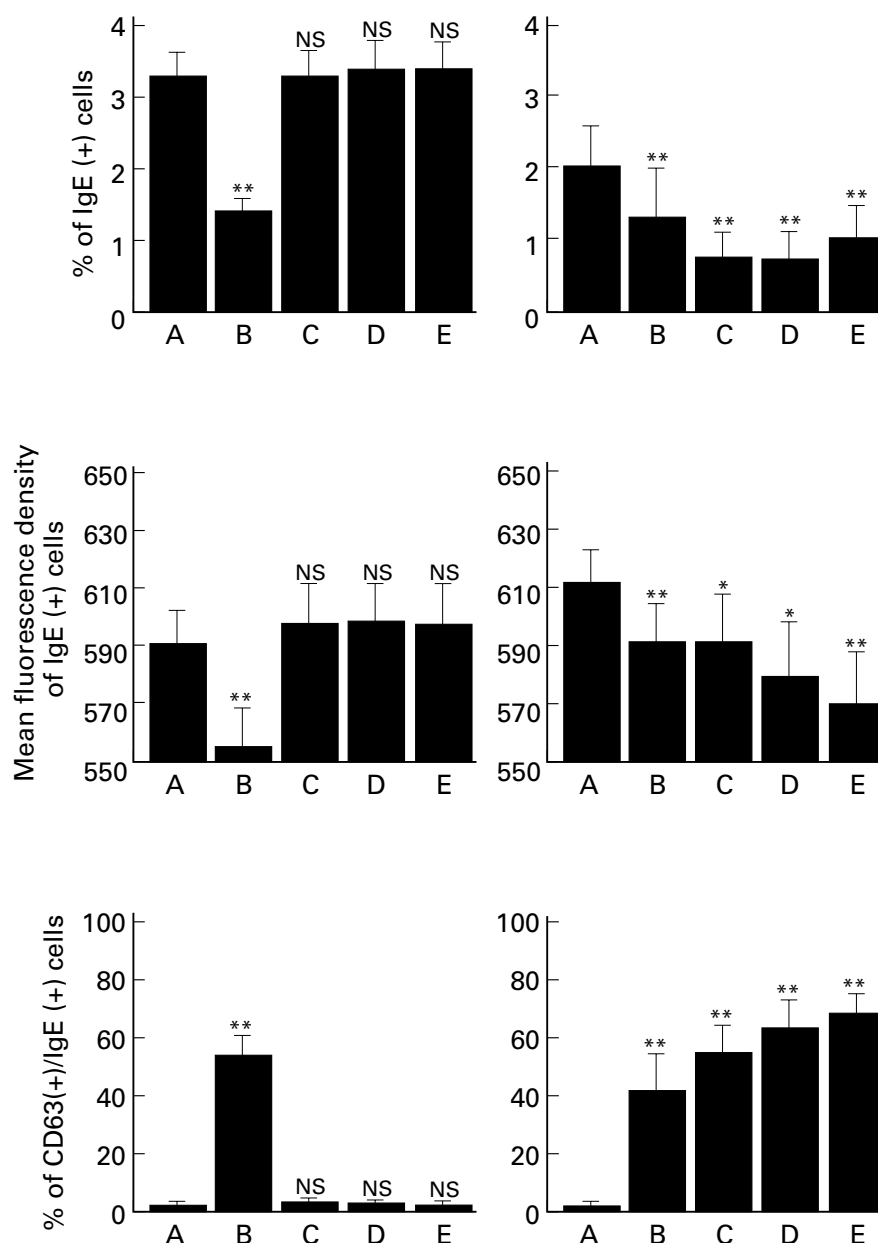


Fig. 1. Variations of the mean percentage of the IgE+ cells, mean fluorescence density and mean percentage of the CD63+ cells related to controls ($n = 12$) and allergic patients ($n = 12$). (A = allergen free controls, B = anti-IgE positive control and C, D, E =, respectively, 1 $\mu\text{g/mL}$, 0.2 $\mu\text{g/mL}$ and 0.04 $\mu\text{g/mL}$ of hymenoptera venom). NS = not significant, * $P < 0.05$ and ** $P < 0.01$.

induced by wasp and honey bee venoms lead to the up regulation of the CD 63 marker on the membrane of activated basophils present at a high density, in some patients, on more than 90% of the gated cells. The flow cytometric method described here was based on the double anti-IgE FITC, anti-CD 63 PE labelling, leading to the calculation, by the cytometer, of three main parameters which were: the percentage of IgE+ basophils, the mean intensity of fluorescence of the basophil population and the percentage of CD63+ basophils. As demonstrated by other authors [15,16] for anti-IgE or aero-allergen, basophil activation induced by venoms lead to a decrease of the

number of IgE+ basophils and to a MIF decrease due to membrane events such as receptor capping.

Basophil activation test (BAT) expressed in percentage of CD63+ basophils showed an excellent sensitivity and specificity as compared to the clinical history observed by highly trained clinicians. However no difference of mean maximum activation was observed between the different clinical reaction types.

The different cellular tests used here were performed in the presence of IL3, this cytokine being known to enhance basophil response measured by the release of histamine or LTC4 [17–21]. This effect was observed here for the release

		Allergen dilution		
		1 µg/mL	0,2 µg/mL	0,04 µg/mL
BAT				
Spontaneous activation (%)	0.69 ± 1.30			
Local reaction (%)		66.3 ± 21.0	55.0 ± 30.0	31.1 ± 28.0
Systemic reactions (%)		46.8 ± 25.4	36.1 ± 26.0	19.2 ± 21.0
Anaphylactic shock (%)		58.3 ± 21.0	43.2 ± 27.0	20.9 ± 17.0
HRL				
Spontaneous release (%)	2.4 ± 27.0			
Total histamine content (ng/mL)	59.8 ± 27.0			
Local reaction (%)		44.0 ± 25.0	37.5 ± 22.0	22.8 ± 17.0
Systemic reactions (%)		31.7 ± 25.0	29.7 ± 25.0	16.7 ± 20.0
Anaphylactic shock (%)		22.8 ± 15.6	21.4 ± 14.3	9.90 ± 9.30
LRT				
Spontaneous release (pg/mL)	105 ± 85			
Local reaction (pg/mL)		1700 ± 730	1700 ± 720	790 ± 840
Systemic reactions (pg/mL)		1420 ± 707	990 ± 755	413 ± 600
Anaphylactic shock (pg/mL)		1350 ± 640	1070 ± 700	450 ± 540

Table 2. Results of BAT, HRL and LRT related to the 45 allergic patients (local reactions $n = 11$, systemic reactions $n = 25$ and anaphylactic shocks $n = 9$)

of histamine, greatly enhanced in the presence of 2 ng/mL IL3 without enhancing the spontaneous release, the low mean spontaneous release of histamine being confirmed by the low spontaneous CD63 expression.

The absence of correlation of BAT with specific IgE level is another argument in favour of the absence of relationship between the amount of circulating specific IgE and basophil releasability. Correlation between BAT and HRL was, on the contrary, highly significant, demonstrating the parallelism existing between CD63 expression and the release of histamine. Correlation with LRT was also significant although LTC4 is a newly formed mediator synthesized according to another biochemical pathway.

In agreement with results obtained previously for other allergens types such as aero-allergens and food allergens, the flow cytometric method described here may be considered as a suitable method for venom allergy diagnosis.

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