

Allergen-induced basophil activation: CD63 cell expression detected by flow cytometry in patients allergic to *Dermatophagoides pteronyssinus* and *Lolium perenne*

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Summary

Background In this study, we determined by flow cytometry the percentage of basophils activated after *in vitro* stimulation by allergens and expressing the CD63 marker. The diagnostic reliability of the technique was assessed as well as its correlation with other *in vitro* diagnostic parameters.

Methods Fifty-three patients suffering from asthma and/or allergic rhinitis following sensitization to *Dermatophagoides pteronyssinus* and 51 patients sensitized to *Lolium perenne* were investigated. Twenty-four atopic patients not sensitive to these allergens and 38 healthy subjects were also selected as controls. The basophil activation test determines the percentage of basophils which express CD63 as an activation marker, by means of flow cytometry, after *in vitro* stimulation with allergen, using double labelling with monoclonal antibody anti-CD63–PE and anti-IgE–FITC.

Results No differences in basal values (non-activated control) were found between sensitized patients, atopic controls and healthy controls. On the other hand, sensitized patients showed a significantly higher percentage of activated basophils after stimulation by allergens *in vitro* than both control groups ($P < 0.001$). We found a significant correlation between skin tests and basophil activation tests ($r = 0.72$, $P < 0.001$). We also found a positive and significant correlation between basophil activation tests and histamine release tests ($r = 0.80$, $P < 0.001$), allergen-specific sulphidoleukotriene production ($r = 0.7$, $P < 0.001$) and the occurrence of serum allergen-specific IgE ($r = 0.71$, $P < 0.001$).

Conclusion The basophil activation test is a highly reliable technique in the diagnosis of allergy to inhalant allergens. The sensitivity of the basophil activation test was 93.3%, and its specificity 98.4%, when using a cut-off point of 15% activated basophils as positive result.

Keywords: basophil activation test, CD63, inhalant allergens, *in vitro* allergologic diagnosis

Clinical and Experimental Allergy, Vol. 31, pp. 1007–1013. Submitted 6 March 2000; revised 14 November 2000; accepted 3 January 2001.

Introduction

Basophils constitute a minor fraction of blood leucocytes, approximately $<0.2\%$. However, these cells play an important role in the pathophysiological process of allergic

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and non-allergic inflammation, being able to release potent proinflammatory mediators, such as histamine, sulphido-leukotrienes and others, which contribute to the development of the inflammatory response. Blood basophils carry allergen-specific IgE immunoglobulins bound to high-affinity IgE receptors. For that reason, detection of basophil interaction with allergen *in vitro*, such as microscopic [1] or flow cytometric [2,3] follow up of basophil degranulation, has been used for allergy diagnosis.

CD63 is a protein molecule of 53 kDa expressed on the cytoplasmic granules of various resting cells. It has been shown that human basophils activated *in vitro* by allergen (Ag) or anti-IgE antibody through their high-affinity receptor for IgE, newly express the CD63 marker also on their membrane [4–7]. It is also known that the CD63 marker may be expressed on the cellular membrane of monocytes, macrophages and platelets [8–10].

Up to 1998 there were only scattered published studies mostly by Sainte-Laudy and Sabbah's groups reporting the use of flow cytometry as a novel technique for the diagnosis of allergic diseases [5–7,11–13]. Since then, some other groups have confirmed the interest in this technique [14–22].

In this study we used a newly optimized flow cytometric procedure to assess CD63 expression and specific IgE on basophils isolated from patients sensitized to house dust mites (Der p) and timothy pollen (Lol p). Optimization included mode of cell handling, use of standardized allergens in fluid phase, selection of most suitable monoclonal antibodies and mode of flow cytometric analysis, as described below. We analysed the reliability of this basophil activation test (BAT), establishing the cut-off level that offers the greatest sensitivity and specificity.

Finally, we compared the results of flow cytometry with those of other *in vitro* diagnostic tests, such as specific IgE determination (RAST), histamine release test (HRT) and Ag-specific sulphido-leukotriene production (CAST).

Materials and methods

Patients

Fifty-three patients allergic to *Dermatophagoides pteronyssinus* (40 male/13 female; age 26.92 ± 12.67 years (mean ± SD)) and 51 patients allergic to *Lolium perenne* (29 male/22 female; age 38.43 ± 13.82 years) were selected from clinical history suggestive of rhinitis, asthma or both and occurrence of positive skin tests and allergen-specific serum IgE. The most frequent clinical manifestations of these patients were rhinitis combined with asthma (63.5%). As atopic controls, we recruited 24 patients: 16 male/8 female, age 30.11 ± 16.55 years, with skin tests and specific IgE negative to *D. pteronyssinus* and *L. perenne*,

but presenting with a total IgE level > 100 kU/L. These atopic controls were sensitized to other allergens (molds, animal dander, mites, pollens) than those used in the BAT.

A group of 38 subjects, six male/32 female, age 32.45 ± 17.69 years, with no personal or family history of atopy and negative skin tests to a battery of common allergens, were considered as healthy controls.

Subjects with infectious diseases, connective tissue diseases, cardiopathies, liver or endocrinological or other immunological disease were excluded.

All subjects stopped the administration of any kind of antihistaminic drugs for at least 5 days (6 weeks for astemizol) before blood sampling. When used for treatment, anti-leukotrienes or systemic corticosteroids were stopped for 1 month prior to blood sampling.

Allergenic extracts

We used aqueous sterile filtered standardized extracts of *D. pteronyssinus* and *L. perenne* devoid of any preservatives (Bial Aristegi, Bilbao, Spain) at final concentrations of Lol p 0.175 and 0.043 mg protein/mL, and of Der p 1.4 and 0.35 mg protein/mL, for HRT, Ag-specific sulphido-leukotriene production by cellular allergen stimulation test (CAST) and Ag-induced BAT. Appropriate allergen dilutions were prepared daily from stock solutions kept at 4°C until the expiry date and used in the fluid phase.

In vivo skin tests

Prick tests were performed with *D. pteronyssinus* and *L. perenne* extracts Lol p 14 150 UBE/mL (internal Biological Units based on specific IgE and skin test reactivity) and Der p 112 900 UBE/mL (Bial Aristegui, Bilbao, Spain) using ALK Abelló lancets. We used histamine hydrochloride (10 mg/mL) as positive control and NaCl 0.9% as negative control. The results were read after 15 min. A weal with diameter ≥ 3 mm compared with the negative control was considered positive.

In vitro tests

IgE determinations Specific and total serum IgE determinations were performed by enzyme immune analysis CAP-FEIA (Pharmacia-Upjohn, Uppsala, Sweden), following the manufacturer's instructions.

Histamine release test We followed the fluorometric method described by Siraganian [23], using a Brän-Lubbe autoanalyser, according to the modified method (performed with whole blood) described in previous papers [24,25]. For positive controls, we used polyclonal anti-IgE antibodies

(Tago, Caltag, Burlingame, CA, USA), at a final concentration of 4.2 µg/mL.

The interpretation of the results was done using the following formula: % of Ag-induced histamine release = (histamine in Ag-stimulated supernatant – basal value) / (total histamine – basal value) × 100, where Ag = histamine release in supernatant after antigen stimulus, basal = spontaneous histamine release in supernatant without stimulus, total = total histamine content in blood sample measured after treatment with perchloric acid 2.5%. Specific histamine release >10% was considered as positive; spontaneous release deducted: median 1.04 (interquartile range (IQ) 0.59–1.53).

Basophil activation test The blood was collected in 6 mL ACD tubes (Vacutainer; Becton Dickinson, Meylan Cedex, France) and stored at 4°C; the test was carried out within 24 h of blood sampling. For testing up to three allergens, one ACD tube was required.

The tubes were centrifuged at 4°C, 10 min, at 550 g. Cells from the layer on top of the erythrocytes were collected and centrifuged again at 4°C, 10 min, at 550 g.

The supernatant was decanted and 500 µL stimulation buffer consisting of HEPES calcium buffer (HEPES 20 mM, NaCl 133 mM, KCl 5 mM, CaCl₂ 7 mM, MgCl₂ 3.5 mM, bovine serum albumin (BSA) 1 mg/mL, pH 7.4), containing IL-3 (2 ng/mL, 10 min, 37°C) and 10 µL of heparin (5000 IU/mL) (ROVI, Madrid, Spain) were added in each tube, mixed and gently shaken.

In a polystyrene microtitre U plate (GREINER MICRO-LON, QUIMILABOR, Madrid, Spain) we placed 50 µL of aqueous extracts of *D. pteronyssinus* and *L. perenne* at two different final concentrations: Lol p 0.175 and 0.043 mg protein/mL, and Der p 1.4 and 0.35 mg protein/mL, in the corresponding wells. As a positive control, we used a monoclonal Le27 anti-IgE antibody (Bühlmann, Allschwil, Switzerland) at a final concentration of 1 µL/mL. In order to evaluate basal values without stimulation, we added merely 50 µL of stimulation buffer in another well.

Then 50 µL of the patient cell suspension were placed in each well and the plate was covered with an adhesive plastic sheet. The plates were incubated at 37°C for 40 min, and the reaction was stopped by adding 100 µL of washing buffer consisting of HEPES buffer pH 7.3 without calcium or magnesium but containing EDTA (HEPES 20 mM, NaCl 133 mM, KCl 5 mM, EDTA 0.27 mM). The plates were then centrifuged for 5 min at 4°C, 1000 g. The supernatant was collected and stored at –80°C until the sulphidoleukotriene (sLT) ELISA assay as provided by Cellular Assay Stimulation Test (CAST-ELISA; Bühlmann Laboratories, Allschwil, Switzerland) was performed.

The basophils from the pellet were immediately double-labelled by adding 20 µL of anti-CD63 PE-labelled

antibody (Caltag Labs), in Cytometer Falcon tubes diluted 1:80 and anti-IgE FITC-labelled antibody (Caltag Labs) diluted 1:60 in washing buffer. After incubation for 30 min at 4°C (protected from light exposure), 4 mL of erythrolytic reagent (Ortho-Mune lytic reagent; Ortho Diagnostic System Inc., San Fernando de Henares, Madrid, Spain) were added to each tube and the tubes were left at room temperature for 5 min. The reaction was stopped with 1 mL of washing buffer. After centrifugation for another 5 min at 1000 g the supernatant was decanted and 500 µL washing buffer added to each tube, with gentle shaking before flow cytometric analysis.

Flow cytometric analysis of surface markers was performed at 488 nm on a FACScan flow cytometer equipped with a 15-nW argon ion laser (Becton Dickinson), using CellQuest software. On the histogram defined by forward scatter and side scatter, the initial cell gate was defined by a bit map around lymphocytes. The second gate was defined around cells showing high density anti-IgE label, identifying them as basophils [26]. In each assay at least 500 basophils were assessed. The other parameter analysed on the identified basophils was CD63.

Antigen-specific sulphidoleukotriene production For allergen-induced sulphidoleukotriene (sLT) ELISA quantification, we followed the instructions of the assay's manufacturer (CAST-ELISA; Bühlmann Laboratories, Allschwil, Switzerland) [27].

Statistical analysis

The data were analysed through the statistical program SPSS 9.0. The results of the descriptive study are expressed as medians value and IQs: 25th percentile to 75th percentile for variables without normal distribution and in mean ± standard deviation (SD) in variable with normal distribution. In order to compare the percentage of basophils activated between patients and control groups, the Kruskal–Wallis test for three independent samples was used. Subsequently, samples were compared two by two. The comparison of means between both groups was done by means of the Mann–Whitney *U*-test or Student's *t*-test for non-paired groups and the Wilcoxon test for paired groups. The non-parametric tests were used after performance of the Shapiro–Wilks test to compare the normality of the samples. For evaluating the correlation between BAT, HRT, serum-specific IgE, skin tests and sLT release we used the Spearman test.

To obtain a specific cut-off point for the significant percentage of activated basophils we used a ROC (receiver operating characteristic) curve. From this, we determined the best sensitivity and specificity for the technique,

Table 1. Results of the *in vitro* tests in sensitized patients and both control groups (median; interquartile ranges (IQ) 25–75 percentiles)

Test	Der p allergic patients <i>n</i> = 53 median; (IQ)	Lol p allergic patients <i>n</i> = 51 median; (IQ)	Atopic control <i>n</i> = 24 median; (IQ)	Healthy control <i>n</i> = 38 median; (IQ)
Total IgE (kU/mL)	253; (127–662.5)*	227; (127–520)*	292; (173–1222)	53.74; (15.1–62.3)
Specific IgE to Der p or Lol p (kU/L)	23.3; (4.54–55.95)†	34.9; (11.2–83.9)†	0	0
HRT				
Basal (%)	2; (1.3–2.9)	1.9; (1.1–2.8)	1.8; (1.3–2.2)	1.1; (0.8–2.1)
Anti-IgE (%)	34.9; (12.7–53.8)**	29.6; (10.3–45.8)**	19.2; (4.5–24)	5.71; (3.1–34.6)
Ag (%)	53.7; (24.5–74.6)†	49.1; (22.6–66)†	1.1; (0–2.5)	0; (0–1.9)
BAT				
Basal (%)	0.5; (0–1.9)	1.5; (0.3–4.7)	0.6; (0–2.6)	0.9; (0.5–4.1)
Anti-IgE (%)	58; (29.3–78.5)*	54.5; (27–70)**	48.5; (12.5–59)	23.5; (2.4–57.2)
Ag (%)	67.6; (49–78.5)†	73.5; (44–82.3)†	2; (0.5–3.8)	1.1; (0.2–2.1)
sLT (CAST)				
Basal (pg/mL)	55.6; (4.4–159.9)	93.3; (35–209.5)	48; (4.4–141)	4.4; (4.4–113.8)
Anti-IgE (pg/mL)	2181; (1461–3788)	2427; (1232–5101)	1768; (927–4686)	3151; (2772–6125)
Ag (pg/mL)	1367; (802–2491)†	1432; (568–2406)†	93.2; (18.1–503)	147; (9.55–269.3)

* $P < 0.001$ between patients and non-atopic controls.

** $P < 0.01$ between patients and non-atopic controls.

† $P < 0.001$ between patients and both controls. HRT, Histamine release test; BAT, basophil activation test; sLT, sulphidoleukotriene.

choosing as cut-off point the value that resulted in the greatest sum for both values [28].

Results

Optimization of BAT

Liquid vs. solid-phase results In 20 cases we analysed BAT to inhalant allergens (*D. pteronyssinus* and *L. perenne* (Bial Aristegi, Bilbao, Spain) at final concentrations of Lol p 0.175 mg protein/mL, and of Der p 1.4 mg protein/mL) in parallel: allergen in solid phase, previously coated in polystyrene microtitre U-plates (Greiner Microton), 18 h at room temperature and kept at 4°C; and allergen in liquid phase (freshly prepared as described in Materials and Methods) at the same concentrations. The mean value of basophil activation in solid-phase assay was $42 \pm 12.5\%$ and the mean value of basophil activation in liquid phase was $68.5 \pm 8.2\%$ (median + SD) ($P < 0.05$). The assay was continued with allergens in liquid phase.

Fresh blood compared with blood kept for 24 h We analysed the reproducibility of the BAT technique in 46 cases, in fresh blood and in blood kept for 24 h. The 24 h blood was kept in ACD tubes (Vacutainer; Beckton

Dickinson) at 4°C. The mean values + SD of basophil activation in fresh blood were 1.9 (IQ 0.3–25.5) and in 24 h blood were 3.5 (IQ 0.6–31.7). No significant differences were found between fresh blood and blood kept for 24 h at 4°C.

Allergen concentrations used were chosen based on past experience with HRT [24,25] and CAST assays [29].

Expression of CD63 on activated basophils (BAT)

Basal and anti-IgE response No significant differences were found in the basal basophil activation test (without antigenic stimulus) among the four study groups (Fig. 1) (Table 1).

Upon *in vitro* stimulation with anti-IgE antibody, atopic patients showed significantly more activated basophils than non-atopic controls (Table 1).

Antigen-specific response No significant difference in CD63 expression was found between atopic (median 2%; (IQ 0.5–3.8)) and non-atopic (median 1.1% (IQ 0.2–2.1)) control groups (Fig. 1) (Table 1). We found a significant increase in allergen-specific basophil activation test compared with the basal response, in both patient groups sensitized to *D. pteronyssinus*: median 67.6% (IQ 49–78.5)

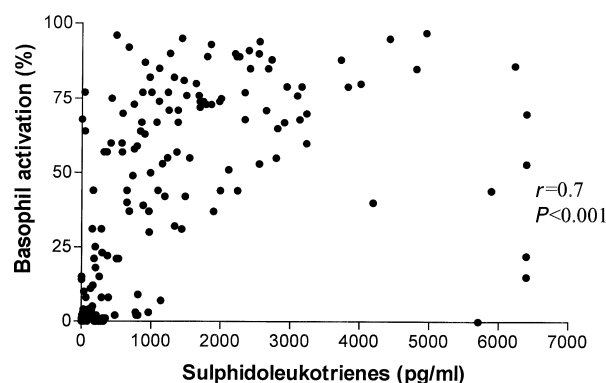


Fig. 1. Basophil activation after *in vitro* stimulation with Der p and Lol p in sensitized patients and controls. Significant differences in Ag-specific response between patients and controls ($P < 0.001$), and between basophil activation test (BAT) Ag and basal in patients ($P < 0.001$).

($P < 0.001$) and to *L. perenne*: median 73.5% (IQ 44–82.3) ($P < 0.001$) compared with controls (Fig. 1) (Table 1).

Basophil activation test reliability Using a ROC curve, we considered 15% of activated basophils as positive result (area under the curve 0.96). Using this cut-off point, we obtained a sensitivity of 93.3% (95% confidence interval (CI) 88.5–99.1%) and a specificity of 98.4% (95% CI 91.3–100%). Predictive positive value was 99% and predictive negative value 89.7%; likelihood ratio for a positive value 58.3 (95% CI 8.25–407) and likelihood ratio for a negative result 0.07 (95% CI 0.09–0.479) compared with the diagnosis performed through clinical history, skin tests and serum specific IgE. The intra-assay variation coefficient between duplicates in 32 cases was 0.66 ± 0.93 , meaning that the procedure is highly reproducible.

Sulphidoleukotriene production (CAST)

Statistically significant differences were found in specific

allergen-induced response between allergen-sensitive patients and both control groups (Table 1).

Correlation between BAT and other in vitro–in vivo tests

A significant positive correlation ($r = 0.72$; $P < 0.001$) was observed between basophil activation after Ag-specific stimulus and skin tests, as well as with specific IgE determination ($r = 0.71$; $P < 0.001$), histamine release test ($r = 0.8$; $P < 0.001$) and Ag-specific sulphidoleukotriene production (CAST) ($r = 0.7$; $P < 0.001$) (Table 2) (Figs 2 and 3).

Discussion

Flow cytometry is an analytic technique suitable to quantify the expression of cellular membrane markers, such as CD63 on basophils. This technique was previously described and developed by Sabbah & Sainte Laudy for allergy diagnosis in 1994–96 [5–8,11–13]. Some technical aspects have been modified by us, such as the use of allergens in fluid phase (with dilutions prepared right before use), and the adjustment of the first stimulation phase to the technique described for the CAST assay (Bühlmann Labs), enabling the determination of CD63 expression and evaluation of sLT production to run simultaneously.

Double-labelling fluorescence allows selection first of the basophil population with monoclonal antibodies marked against IgE or the high-affinity IgE receptor, and then the activated basophils with monoclonal antibodies directed against CD63. Other authors [14] use Ab anti-CD45 as an additional marker. In our experience, this addition does not improve the results (results not shown). Our finding is supported by Gane *et al.* [26], who showed that during basophil activation after allergen stimulation, an up-regulation of CD45 expression also takes place.

In our procedure, we first delimit the area of lymphocyte

Table 2. Correlation between basophil activation test (BAT) and other diagnostic tests

			Sulphidoleukotriene (CAST)	Histamine release (HR)	Specific IgE	Skin tests
BAT	Der pt	r	0.69	0.84	0.73	0.73
	p	< 0.001	< 0.001	< 0.001	< 0.001	
BAT	Lol p	r	0.69	0.75	0.67	0.7
	p	< 0.001	< 0.001	< 0.001	< 0.001	
BAT	Total	r	0.7	0.8	0.71	0.72
	p	< 0.001	< 0.001	< 0.001	< 0.001	

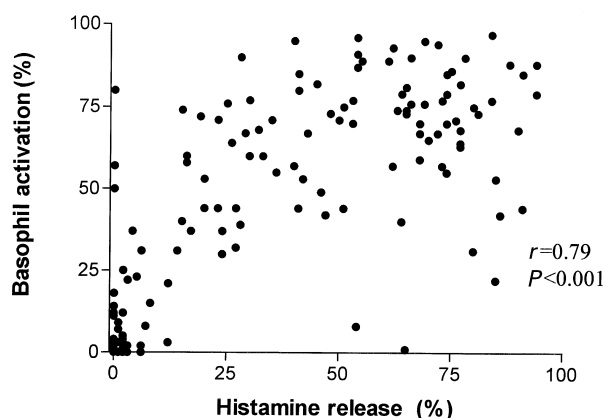


Fig. 2. Correlation between basophil activation test and histamine release test.

population, and by using two markers (anti-IgE labelled with FITC or Ab against high-affinity receptors for IgE and anti-CD63 labelled with PE as activation marker) we evaluate quite specifically the population of activated basophils. The number of basophils evaluated is ≥ 500 and represents a higher number of cells than those investigated in previous reports [12].

In patients sensitive to house dust mites or timothy pollen, our results showed a significant increase in the percentage of activated basophils, after specific Ag stimulation, compared with the basal non-stimulated state. This supports the claim that CD63 expression reflects basophil activation in patients with allergy to inhalant allergens. Our results show higher percentages of activated basophils than those reported by other authors [14], which may reflect better test conditions. Indeed, in preliminary experiments we had observed that the quality of the allergens used, their fresh preparation in fluid phase and

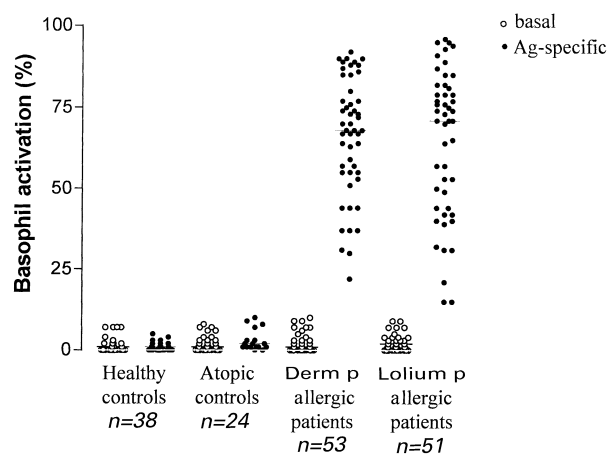


Fig. 3. Correlation between basophil activation test and sulphido-leukotriene production (CAST).

optimization of stimulation conditions all influenced the results.

No previous study has compared the basophil activation test with the whole range of cellular and other diagnostic assays available and has evaluated the overall clinical sensitivity and specificity of the technique and its optimal cut-off point in terms of diagnostic reliability for inhalant allergens. We found that using a positivity cut-off point of 15% activated basophils offers a high sensitivity (93.3%), specificity (98.4%) and likelihood values. Sainte Laudy *et al.* [11] chose as cut-off point an increase of 5% in the number of activated basophils compared with the basal response, but did not indicate the basis for such cut-off selection.

The good correlation observed between this technique and the rest of the *in vivo* and *in vitro* diagnostic techniques makes us consider the basophil activation test a highly reliable procedure for detection of IgE-mediated allergy to inhalant allergens. As shown by other studies in allergy to Betalactam antibiotics [30,31], latex [19,32] and food allergens [13,33,34], the flow cytometric analysis of activated basophils following *in vitro* stimulation by allergens is a highly efficient diagnostic technique in allergy.

Acknowledgements

We thank Ms Sonia Ariz her technical assistance and Ms Gema Betelu for her help in the preparation of the manuscript.

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