

Analysis of basophil activation by flow cytometry in pediatric house dust mite allergy

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Detection of allergen-induced basophil activation by flow cytometry has been shown to be a useful tool for allergy diagnosis. The aim of this study was to assess the potential of this technique for the diagnosis of pediatric house dust mite allergy. Quantification of total and specific IgE and basophil activation test were performed to evaluate mite allergic ($n = 24$), atopic ($n = 23$), and non-allergic children ($n = 9$). Allergen-induced basophil activation was detected as a CD63-upregulation. Receiver operating characteristics (ROC) curve analysis was performed to calculate the optimal cut-off value of activated basophils discriminating mite allergic and non-allergic children. ROC curve analysis yielded a threshold value of 18% activated basophils when mite-sensitized and atopic children were studied [area under the curve (AUC) = 0.99, 95% confidence interval (CI) = 0.97–1.01, $p < 0.001$] with a sensitivity and specificity of 96% for 16 $\mu\text{g}/\text{ml}$ mite extract. Analysis of the data obtained with 1.6 $\mu\text{g}/\text{ml}$ mite extract defined a cut-off value of 8% activated basophils (AUC = 0.96, 95% CI = 0.91–1.01; $p < 0.001$) with a sensitivity of 82% and specificity of 100%. Comparison between mite allergic and non-allergic children produced a cut-off of 8% activated basophils (AUC = 1.0) with 16 $\mu\text{g}/\text{ml}$ allergen extract and a sensitivity and specificity of 100%. The same threshold and specificity values were obtained with 1.6 $\mu\text{g}/\text{ml}$ extract (AUC = 97%, 95% CI = 0.92–1.02; $p < 0.001$) but sensitivity decreased to 83%. Two atopic children showed negative skin prick and basophil activation tests and high specific IgE (> 43 kU/l) values for *Dermatophagoides pteronyssinus* allergen. They also showed positive prick (wheal diameter > 1.0 cm) and basophil activation ($> 87\%$) tests and high specific IgE (> 100 kU/l) with shrimp allergen. Shrimp sensitization was demonstrated by high levels of Pen a 1-specific IgE (> 100 kU/l). Cross-reactivity between mite and shrimp was confirmed by fluorescence enzyme immunoassay (FEIA-CAP) inhibition study in these two cases. This study demonstrated that the analysis of allergen-induced CD63 upregulation by flow cytometry is a reliable tool for diagnosis of mite allergy in pediatric patients, with sensitivity similar to routine diagnostic tests and a higher specificity. Furthermore, this method can provide additional information in case of disagreement between *in vivo* and *in vitro* test results.

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House dust mites are important indoor allergens in the induction of rhinitis and asthma (1, 2). Diagnosis is based on the patient's clinical history, skin prick tests, and specific IgE quantification. Functional *in vitro* assays detecting mediators release have been also used for the

diagnosis of mite allergy (3–7). These assays have not become a routine practice in clinical laboratory because they are cost and time consuming procedures. Because of these issues, efforts have been made at finding a new approach to study allergic patients (8, 9).

Basophils are one of the effector cell involved in allergic reactions. These cells bear IgE bound by their membrane Fc ϵ type I receptors. On challenge with a specific allergen, basophils release mediators and change the phenotype. Change in phenotype is because the expression of several basophil surface molecules are modulated during basophil activation (10–13) and these changes are easily analyzed with a flow cytometer. Flow cytometry allowed to study minor cellular populations, as basophils, and analyzed single cell-based parameter. Among modulated molecules of the basophils, CD63 is the most widely used in the study of allergy (14, 15). This protein is a member of the transmembrane-4 superfamily and it is anchored in the intracellular granules. CD63 is a dim cellular marker in resting basophils that becomes a bright marker when basophils are activated with a specific allergen, as a result of fusion between granules with plasmatic membrane. It has been shown that basophil CD63 upregulation parallels with histamine release (16).

In this study, we assessed the basophil activation test for the diagnosis of house dust mite (*Dermatophagoides pteronyssinus*) allergy in pediatric patients. Two cases of disagreement between skin prick test and specific IgE quantification that appeared during the study were investigated.

Patients and methods

Twenty-four children with clinical history suggestive of allergy to mites, showing rhinitis and/or asthma and a positive skin prick test with *Dermatophagoides pteronyssinus* were selected for the study. Atopic pediatric patients and non-allergic children were also included as controls. The atopic control group (n = 23) was defined by a negative prick test to mites and a positive skin test to any of the common allergens (molds, pollens, cat, dog, and cockroach) found in our urban area (17). The non-allergic group (n = 9) had no history of allergy and negative skin prick tests to the common allergens tested.

After obtaining informed consent from the patient's parents, prick tests, serum IgE quantification and basophil activation test were performed. Skin prick test was carried out with commercial allergen extracts (Bial Aristegui, Bilbao, Spain). Both positive (glycerinated histamine 10 mg/ml) and negative controls (glycerosaline solution) were used. Skin prick tests were considered positive if wheal diameter was > 3 mm.

Quantification of total and specific IgE

Serum-specific IgE determinations were performed by FEIA-CAP (UniCAP, Pharmacia-Upjohn, Uppsala, Sweden) according to the manufacturer's instructions. The detection range was 0.35–100 kU/l. Results >0.35 kU/l were considered positive. Total IgE was quantified with an enzyme immunoassay (Access Total IgE; Beckman Coulter Inc., Fullerton, CA, USA). The detection range was 0.25–3000 IU/ml.

Basophil activation test

Basophil activation test was performed as previously described (18). One hundred microliter of sample was incubated for 15 min at 37°C with *D. pteronyssinus* commercial extract (16 µg/ml, 1.6 µg/ml, 0.16 µg/ml final concentrations; Bial Aristegui). In two cases, basophil activation test was also performed with 1 µg/ml and 0.1 µg/ml *Blatta orientalis*, 1.5 µg/ml and 0.15 µg/ml *Anisakis simplex* and 1 µg/ml and 0.1 µg/ml *Penaeus aztecus* (Bial Aristegui). Negative and positive controls were performed by incubating blood without allergen and 10 µl anti-IgE (10 µg/ml Pharmingen; BD Biosciences, Erembodegem, Belgium) respectively.

To analyze basophil activation, cells were stained with 20 µl CD63-FITC/CD123-PE/HLA-DR-PerCP reagent mixture (BD Biosciences) for 20 min in the dark on ice. Then, samples were incubated with 2 ml FACS Lysing solution (BD Biosciences) for 15 min at room temperature. After centrifugation (300 g, 5 min) and washing with PBS, cells were resuspended with 300 µl 1% paraformaldehyde and acquired in a FACScan flow cytometer (BD Biosciences).

To analyze the percentage of allergen-activated cells, basophils were gated and CD63 expression was detected as described (18). Percentage of activated basophils was calculated by subtracting spontaneous CD63 expression (negative control) from the data obtained with allergen stimulation.

Statistics

Statistical analysis was performed with SPSS 12.0 software (Chicago, IL, USA). Shapiro-Wilks test was performed to analyze normality of parameters. Median and interquartile range (IQR) described continuous variables. Intergroup comparisons of continuous variables were performed with the Mann-Whitney *U*-test and bivariate correlation was analyzed with the Spearman's rank test. Receiver operating characteristics (ROC) curve analysis was performed to calculate

the optimal cut-off value of activated basophils corresponding to the greatest sum for specificity and sensitivity values (19).

Results

The aim of this work was the assessment of basophil activation detection by flow cytometry for the diagnosis of the pediatric mite allergy. Three groups of children were studied. There were no differences between the age of the allergic children (9, IQR = 6–12 yr) and the atopic (6, IQR = 5–10 yr) and non-allergic controls (6, IQR = 5–9 yr). Comparison of total serum IgE values showed a higher concentration in patients than in healthy controls (Table 1; $p < 0.01$). Determination of *D. pteronyssinus*-specific IgE showed positive values in sensitized children and negative values in non-allergic patients (Table 1). Atopic patients showed negative values for specific IgE but two children had 0.60 kU/l (class 1) and 0.72 kU/l (class 2) and another two children showed very high levels of *D. pteronyssinus*-specific IgE (43.3 and 44.9 kU/l, both class 4). These two patients were further studied in an attempt to clarify the clear disagreement between prick test and specific IgE value. As a result of these false positives, mite-specific IgE quantification showed a sensitivity of 100% and a specificity of 85%.

The analysis of allergen concentration curves for the basophil activation test showed almost no change in CD63 upregulation in control subjects. On the contrary, basophil activation test was dose dependent in the mite allergic children (Fig. 1). Comparison of basophil activation test between allergic and controls showed that there were no differences in both basal CD63 expression and anti-IgE induced upregulation of CD63 expression. Significant differences between study groups in basophil activation test are shown in Table 1.

Receiver operating characteristics curve analysis was performed to determine the cut-off value

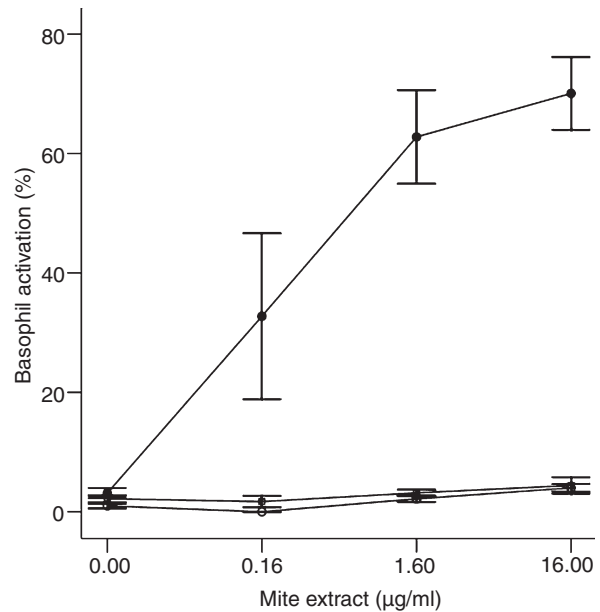


Fig. 1. *Dermatophagoides pteronyssinus*-induced basophil activation in sensitized children and controls. Blood samples from children with mite allergy (n = 24; solid circles), atopic children (n = 23; open squares) and non-allergic children (n = 9; open circles) were assayed with three mite extract concentrations. Results are expressed as percentages of CD63⁺ basophils above basal values (mean ± standard error).

of the percentage of activated basophils between mite allergic children and controls. Comparison between mites sensitized and atopic children yielded a threshold value of 18% activated basophils [area under the curve (AUC) = 0.99, 95% confidence interval (CI) = 0.97–1.01, $p < 0.001$] with a sensitivity and specificity of 96% for 16 µg/ml allergen extract. Analysis of the data obtained with 1.6 µg/ml allergen extract defined a cut-off value of 8% activated basophils (AUC = 0.96, 95% CI = 0.91–1.01; $p < 0.001$) with a sensitivity of 82% and specificity of 100%. Comparison between mite allergic and non-allergic children produced a cut-off of 8% activated basophils (AUC = 1.0) with 16 µg/ml allergen extract and a sensitivity and specificity of

Table 1. Serum IgE values and percentage of activated basophils (median, IQR) in mite sensitized children and atopic and healthy controls

	<i>Dermatophagoides pteronyssinus</i> allergic patients (n = 24)	Atopic controls (n = 23)	Non-allergic controls (n = 9)
Total IgE (IU/ml)	276 (140–705)*	158 (25–647)*	6 (4–28)
Specific IgE to <i>D. pteronyssinus</i> (kU/l)	11.2 (1.7–49.6)**	<0.35	<0.35
Activated basophils (%)			
Basal	2.0 (1.0–3.2)	1.4 (0.6–3.0)	0.5 (0.2–1.2)
Anti-IgE	84.0 (58.0–92.5)	71.0 (48.6–87.0)	53.6 (40.5–83.5)
<i>D. pteronyssinus</i> 16 µg/ml	78.0 (30.0–94.2)**	2.0 (0.0–6.0)	3.5 (3.0–5.9)
<i>D. pteronyssinus</i> 1.6 µg/ml	71.5 (7.5–94.5)**	1.0 (0.0–2.0)	0.9 (0.2–1.8)

* $p < 0.01$ between mite allergic and atopic children and healthy controls.

** $p < 0.001$ between mite allergic children and both control groups.

100%. The same threshold and specificity values were obtained with 1.6 µg/ml extract (AUC = 97%, 95% CI = 0.92–1.02; $p < 0.001$) but sensitivity decreased to 83%.

The analysis of the association between the functional test and the serologic data in the mite allergic children showed that the percentage of activated basophils significantly correlated with specific IgE values ($r = 0.47$, $p = 0.02$) but did not with total IgE values ($p = 0.80$). Furthermore, an association between basophil activation and age ($r = 0.43$, $p = 0.04$) was found.

As mentioned above, two atopic children (positive skin prick test for cockroach and negative for pollen and mold) showed a negative skin prick test and high specific IgE values for *D. pteronyssinus* allergen. They were 11- and 9-yr-old and had a moderate to persistent asthma. Prick test, specific IgE quantification and basophil activation test were performed with cockroach (*Blatta orientalis*), *Anisakis simplex*, and shrimp (*P. aztecus*; Table 2). Assays with shrimp allergen yielded the highest values for prick test (wheal diameter > 1.0 cm), specific IgE (> 100 kU/l) and basophil activation (> 87%). Prick test and basophil activation test were only positive for cockroach and shrimp allergens, while positive specific IgE values were found for all allergens tested (Table 2). As the major allergen of shrimp is tropomyosin (Pen a 1; 20), recombinant Pen a 1-specific IgE was also determined by FEIA-CAP and values > 100 kU/l were obtained (Table 2). To test if cross-reactivity was involved in the positive specific IgE values for mite, *Anisakis* and cockroach, FEIA-CAP inhibition assays were performed with shrimp. Shrimp extract inhibited mite-specific IgE by 99%, *Anisakis*-specific IgE by 96% and cockroach-specific IgE by 98%.

Table 2. Basophil activation test in two tropomyosin sensitized pediatric patients

	Allergen				
	Mite	Shrimp	Anisakis	Cockroach	rPen a 1
Patient 1					
Specific IgE (kU/l)	44.9	>100	27.7	80.4	>100
Prick test	–	+	–	+	n.d.
Basophil activation (%)	9	88	1	16	n.d.
Patient 2					
Specific IgE (kU/l)	43.3	>100	28.1	89.8	>100
Prick test	–	+	–	+	n.d.
Basophil activation (%)	4	96	2	40	n.d.

n.d., not determined.

Discussion

Basophil activation with specific allergen has proven to be useful for diagnosis of pollen, latex, food, Hymenoptera venom, and drug allergies (15). In this study, dose–response curves analyses showed that basophils from mite-sensitized children were activated with allergen concentrations ranging three log scales, while control basophils remained unchanged. Comparison between mite-allergic, atopic and non-allergic children showed that maximal differences in percentages of activated basophils were obtained upon stimulation with 1.6 µg/ml and 16 µg/ml mite extract. ROC analysis yielded similar cut-off values of activated basophils for pediatric patients than those previously reported for adult patients (21, 22). Taking into account the specificity and sensitivity values obtained with the two allergen concentrations, basophil activation test reached a sensitivity of 96% (16 µg/ml; threshold of 18%) and a specificity of 100% (1.6 µg/ml, threshold of 8%). Furthermore, analysis of CD63 upregulation induced with allergen discriminated between mite-allergic and non-allergic children with a sensitivity and specificity of 100%. Therefore, basophil activation test is a highly efficient test for diagnosis of pediatric mite allergy as it has been reported for adult patients (22).

Specific IgE quantification and basophil activation test showed a similar sensitivity (100% vs. 96%) but specificity of basophil activation test (100%) was higher than that of specific IgE measurement (85%) when comparing mite allergic and atopic children. Clear higher specificity for basophil activation test was also found in other IgE-mediated hypersensitivity as pollen allergy (23), latex allergy (24), or Hymenoptera venom allergy (25).

Two atopic children not clinically sensitized to mites showed a disagreement between mite-specific IgE and prick test results. They had high reactivity to shrimp allergen determined by specific IgE determination, prick test and basophil activation test and high values to r Pen a 1, the major allergen in shrimp (20). The shrimp muscle protein tropomyosin has been suggested to be an invertebrate pan-allergen and to be involved in clinical cross-sensitivity among invertebrates (26). In fact, IgE reactivity to shrimp has been showed in unexposed population of subjects allergic to mite and/or cockroaches (27) and, conversely, a study has suggested that sensitization to shrimp tropomyosin can cause allergy to mite and *B. germanica* (26). Since major allergens in mite (28), cockroach (29) and *A. simplex* (30)

are proteins other than tropomyosin, only some decrease in the specific IgE values should be observed upon inhibition with shrimp. However, inhibition assays indicated that the presence of specific IgE to these allergens was responsible for cross-reactivity to shrimp allergen in these two atopic children. As the two children have not eaten shellfish for 1 yr at least and they show allergic symptoms, it is tempting to think that cockroach allergen (the other allergen showing positive skin prick and basophil activation tests) is responsible for their asthma.

Our study demonstrated that the analysis of allergen-induced CD63 upregulation by flow cytometry is a reliable tool for diagnosis of mite allergy in pediatric patients, with a sensitivity similar to routine diagnostic tests and a higher specificity than that of specific IgE determination. Furthermore, this method can provide additional information in case of disagreement between *in vivo* and *in vitro* test results.

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