ORIGINAL ARTICLE

A New Variant of the Basophil Activation Test for Allergen-Induced Basophil CD63 Upregulation. The Effect of Cetirizine

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Abstract

Objective: The aim of our study was to determine the diagnostic usefulness of a newly developed basophil activation test (BAT) in patients allergic to *Dermatophagoides pteronyssinus* and pollens. We also analyzed the influence of cetirizine on CD63 upregulation. This popular antihistamine strongly inhibits skin tests, but its impact on BAT sensitivity remains unknown and deserves at least preliminary determination.
Methods: The study sample comprised 22 patients allergic to house dust mite and pollens and 19 healthy controls. All participants underwent skin prick testing and the newly developed flow-cytometric basophil activation test. The protocol for allergen-induced basophil CD63 upregulation consisted of whole blood samples that were processed and stained with anti-CCR3/CD63 antibodies added to the buffer at the beginning of stimulation. Skin prick tests and BAT were performed twice – before and 2 hours after ingestion of 10 mg of cetirizine.
Results: The new BAT is characterized by its short processing time, easy basophil gating, and strong CD63 upregulation with very high sensitivity and excellent specificity. Our results suggest that allergen-induced CD63 upregulation by higher doses of allergens is not inhibited 2 hours after administration of cetirizine (unlike skin prick tests).
Conclusion: The BAT is a very useful and precise method for the diagnosis of allergy to aeroallergens. It is not influenced by cetirizine.

Key words: Basophil. CD63. Allergy. Flow cytometry.

Resumen

Objetivo: El objetivo de nuestro estudio fue determinar la utilidad diagnóstica de un recientemente desarrollado test de activación del basófilo (TAB) en pacientes alérgicos a *Dermatophagoides pteronyssinus* y pólenes. También analizamos la influencia de la cetirizina en la regulación al alza del CD63. Este popular antihistamínico inhibe potentemente las pruebas cutáneas, pero su efecto en la sensibilidad del TAB permanece sin dilucidar.
Métodos: La muestra del estudio comprendió 22 pacientes alérgicos a ácaros del polvo de casa y polen así como 19 controles sanos. Todos los participantes se sometieron a pruebas cutáneas y al nuevo TAB. El protocolo para la regulación al alza del CD63 basofílico inducido por alérgeno consistió en muestras de sangre completa que se procesaron y tiñeron con anticuerpos anti-CCR3/CD63 añadido al búfer al principio de la estimulación. Las pruebas cutáneas y el TAB se realizaron antes y 2 horas después de la toma de 10 mg de cetirizina.
Resultados: El nuevo TAB se caracterizó por su corto tiempo de procesado, la fácil selección de los basófilos, y la fuerte regulación al alza del CD63 con una alta sensibilidad y excelente especificidad. Nuestros resultados sugieren que la regulación al alza del CD63 inducido por alérgeno por altas dosis de alérgeno no se inhibe después 2 horas tras la administración de cetirizina (a diferencia de las pruebas cutáneas).
Conclusión: El TAB es un método útil y preciso para el diagnóstico de la alergia a aeroalérgenos. No está influenciado por la cetirizina.

Palabras clave: Basófilo. CD63. Alergia. Citometría de flujo.

Introduction

Diagnosis of allergy requires confirmation of clinical data on patient immune status. In the case of immediate-type of allergic reactions, skin testing with the suspected allergens and/or allergen-specific immunoglobulin (Ig) E determination is often successful. However, these procedures sometimes fail and the culprit allergen cannot be easily detected. In such cases, provocation tests can be considered, although they are usually time-consuming and can lead to severe systemic reactions. Therefore, other sensitive laboratory tests are necessary to establish a diagnosis.

In 1994 Sainte-Laudy et al [1] developed the first flow cytometry assay, the basophil activation test (BAT), which was based on identification of CD63 on the surface of stimulated basophils. Many studies confirmed this first observation and the usefulness of the assay in the diagnosis of allergy to aeroallergens, food allergens, Hymenoptera, latex, and drug allergens [2-14]. Most of these studies used anti-IgE assays to identify the allergen; however, the relatively lower sensitivity observed in the studies on drug allergy means that assays to confirm a diagnosis of drug allergy had to be reviewed in detail.

In the search for improved sensitivity, Sainte-Laudy et al [15] showed that downregulation of IgE was more sensitive than upregulation of CD63 in the diagnosis of ß-lactam allergy. The sensitivity of BAT can also be improved by identification of basophils. Some investigators have analyzed basophil gating using the combination of anti-CD123 and anti-HLADR antibodies, or anti-CD203c antibody alone, or a combination of anti-CRTH2 and anti-CD3, although these techniques have not been compared to date [16-19].

The aim of the present study was to determine the sensitivity and specificity of a newly developed BAT in patients allergic to *Dermatophagoides pteronyssinus* and grass pollens. The assay combines anti-CCR3 antibody (for basophil identification) and anti-CD63 antibody (to determine the degree of basophil activation). CCR3 is constitutively expressed on eosinophils and basophils, and flow cytometry gives satisfactory results in basophil counting [20]. Basophils can then be easily gated on the basis of their high CCR3 expression ($CCR3^{high}$) and low side scatter (SSC^{low}) position (Figure 1).

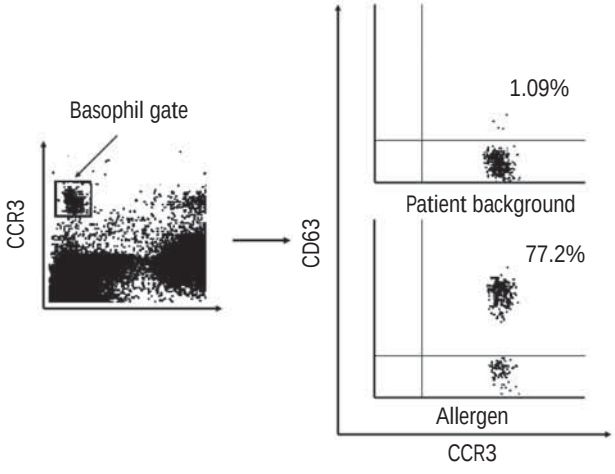

Figure 1. Gating strategy.

This combination has already proven successful only in one study focusing on in vitro diagnosis of allergy to muscle relaxants, although anti-CD45 antibody was also used in the first phase of basophil gating [6]. In the present study, we used the combination of antibodies and a shorter cell stimulation time.

The influence of popular antiallergic medication on basophil CD63 upregulation is unknown. Antihistamines require special attention, as they can inhibit skin tests. Consequently, a laboratory test not influenced by this medication might have an important advantage over skin testing in some patients. Therefore, we also performed an open-label, pilot study to determine the effect of cetirizine on basophil CD63 upregulation.

Methods

Patients

The study population comprised 22 patients (9 men and 13 women) aged 21 to 40 years (mean age, 23 years) with allergic rhinitis and/or atopic asthma who had positive skin prick test results to *D pteronyssinus* (16 patients) and/or a 6-grass mix (BAG-GX1, Bühlmann Laboratories AG, Schönenbuch, Switzerland) (12 patients).

Controls

The control group comprised healthy individuals (5 men and 14 women) aged 22 to 28 years (mean age, 23 years) with negative skin prick test results.

Study Design

The first blood sample was taken between 8.30 AM and 9.00 AM and the skin tests performed. A few minutes later, 1 dose of cetirizine (10 mg) was administered, followed 2 hours later by the second blood sample and skin testing.

Skin Prick Tests

Skin prick tests were performed according to the standard procedure using reagents supplied by Allergopharma Joachim Ganzer KG (Reinbek, Germany). The tests were read after 10 minutes for histamine solution and 15 minutes for allergens. All wheal sizes were expressed as the mean of the longest diameter and midpoint perpendicular diameter. A skin prick test result was considered positive if the mean wheal diameter was ≥3 mm.

Basophil CD63 Expression

Expression of CD63 was determined using FLOW2 CAST kits (Bühlmann Laboratories AG) according to the manufacturer's instructions. Blood specimens were collected using K-EDTA venipuncture tubes (Sarstedt AG & Co, Nümbrecht, Germany), and cell stimulation was performed immediately using BD Falcon Round Bottom Tubes (BD Biosciences Pharmingen, San Diego, California, USA). Testing was performed for each patient using separate tubes as follows:

patient background (PB), stimulation control with anti-FcεRI antibody (PC1), stimulation control with formyl-Met-Leu-Phe (fMLP) (PC2), and allergens at the 2 final concentrations of 250 ng/mL and 25 ng/mL for *D pteronyssinus* (D1) and 25 ng/mL and 2.5 ng/mL for the 6-grass mix.

Cell Stimulation and Staining

Fifty microliters of the corresponding stimulus was added to each tube as follows: PB tube, stimulation buffer (patient background); PC1 tube, stimulation control anti-FcεRI antibody; PC2 tube, stimulation control–fMLP; allergen tubes, allergen solution. In the following step, 100 µL of stimulation buffer and 50 µL of whole blood was added to each tube and gently mixed. Twenty microliters of staining reagent was then added to each tube and mixed gently before being covered and incubated for 15 minutes at 37°C in a water bath.

Lysing and Cell Acquisition

Stimulation was terminated by addition of 2 mL of the prewarmed (18-28°C) lysing reagent and after mixing tubes were incubated at room temperature for 5-10 minutes. After centrifugation (5 minutes at 500*g*), supernatants were decanted using blotting paper. Cell pellets were resuspended with 300 µL of wash buffer and gently vortexed. A total of 150 000 cells were acquired per sample using the FACScan flow cytometer (BD Biosciences Pharmingen).

Data Analysis

Data were analyzed using CellQuest flow cytometry analysis software (BD Biosciences Pharmingen) according to instructions from Bühlmann Laboratories AG. In the first step, a gate (R1) was set by including the entire basophil population ($CCR3^{high}$) with low side scatter (SSC^{low}). Eosinophils are located on the upper right and can be excluded due to their SSC^{high} position. In the second step, calculation of the percentage of CD63-positive cells (brightly fluorescent fluorescein isothiocyanate) was determined by comparing its number to the total amount gated in R1 (Figure 1). The results were presented as percentages after subtracting patient background values. The cut-off values for allergen stimulation positivity and specificity were determined using 2-graph receiver operating characteristic (ROC) curves [21,22] and compared to the manufacturer's recommended value of 15%. For positive controls, the cutoff value was set at 10% (according to the manufacturer's recommendations).

Statistics

Data distributions were checked for normality using the χ^2 test. Depending on the results obtained, a nonparametric Wilcoxon test was used for further analysis.

Ethics

Our study was performed in accordance with the Declaration of Helsinki 1975 (revised 1983) and was approved by the Ethics Committee of our institution.

Results

Skin Prick Tests

Wheal diameter in the study group (n=22) after the skin prick test with histamine ranged from 3 mm to 7.5 mm (median diameter, 3 mm). Two hours after intake of cetirizine, the reduction in diameter was statistically significant (median diameter, 1 mm [min, 0 mm; max, 7.5 mm, *P*<.000001); in the control group, wheal diameter ranged from 2 mm to 8 mm (median diameter, 4 mm), and 2 hours after intake of cetirizine, the reduction in diameter was statistically significant (median diameter, 1 mm [min, 0 mm; max, 2.5 mm], *P*=.000132). The result of the skin prick test with *D pteronyssinus* in patients allergic to this allergen (n=16) was positive in every case (median diameter, 7.75 mm [min, 3 mm; max, 16 mm), and 2 hours after intake of cetirizine it was significantly lower than at baseline (median diameter, 2.75 mm [min, 0 mm; max, 10.5 mm], *P*=.000438). Analysis of personal data revealed cetirizine-induced test inhibition in 50% of patients (mean diameter ≤3 mm). Skin prick testing with the 6-grass mix in grass-allergic patients (n=12) was positive in every case (median, 5.75 mm [min, 4 mm; max, 17 mm]) and 2 hours after intake of cetirizine

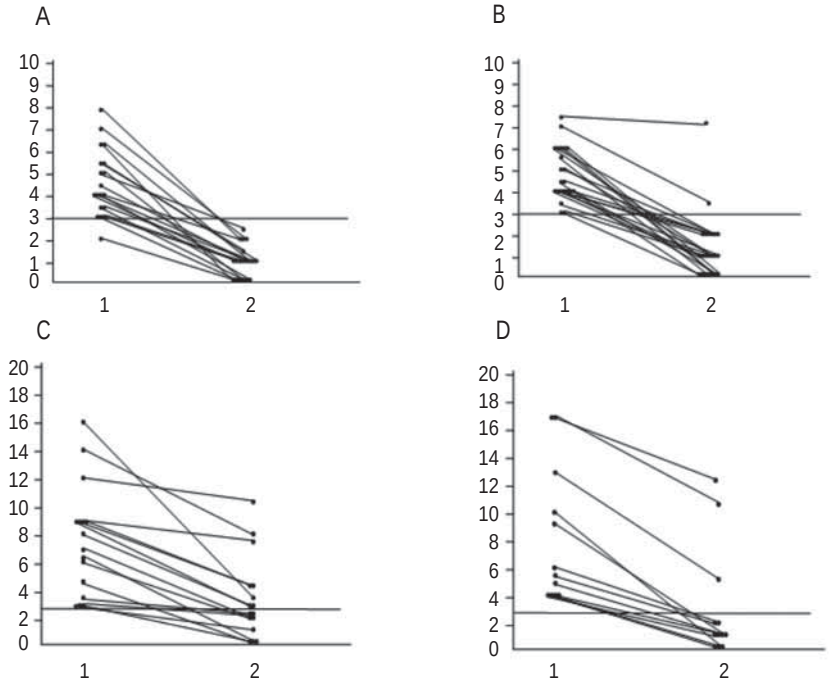

Figure 2. Skin test results with histamine (mm diameter) in the control group (A), in the whole patients group (B), with relevant allergens in patients sensitized to *Dermatophagoides pteronyssinus* (C), and in those sensitized to the 6-grass mix (D), before (1) and 2 hours after (2) 10 mg of cetirizine.

it was significantly lower (median, 1 mm [min, 0 mm; max, 12.5 mm], *P*=.002218). Analysis of personal data indicated cetirizine-induced test inhibition in 75% of the patients (median diameter ≤3 mm). In the control group, skin prick test results with these allergens were negative both before and after intake of cetirizine (Figure 2).

Basophil CD63 Expression

Basophil number: The number of basophils in the population tested (n=41) ranged from 169 to 1845 cells (median, 607 cells).

PB: In the population tested, PB values were similar: mean, 0.9% (min, 0%; max, 15.7%).

Positive controls: In the healthy controls, anti-FcεRI stimulation resulted in very intense expression of basophil CD63. The median percentage of CD63-positive basophils reached 69.6% (min, 23.9%; max, 95.3%). Two hours after cetirizine intake, the results of cell stimulation were significantly lower. The median percentage of CD63-positive cells was 57.3% (min, 17.5%; max, 94.4% [*P*=.002543]). This stimulation was 100% sensitive before and after intake of cetirizine. After fMLP stimulation, the median percentage of CD63-positive cells was 34.1% (min, 4.76%; max, 75.7%) and 2 hours later these values remained unchanged (median, 27.3% [min, 6.4%; max, 70.3%]; *P*=.067099). This stimulation was 94.73% sensitive before intake and 89.47% sensitive after (Figure 3).

In the patient group, anti-FcεRI stimulation also resulted in an intense increase in expression of basophil CD63 (median, 87.85% [min, 13.3%; max, 95.4%]). Two hours after cetirizine intake, the results of cell stimulation were significantly lower. The median percentage of CD63-positive cells was 80.85% (min, 12.2%; max, 94.4% [*P*=.000075]). This stimulation was 100% sensitive before and after cetirizine intake. After fMLP stimulation, the median percentage of CD63-positive cells was 44.3% (min, 6.8%; max, 81.9%) and 2 hours later these values remained unchanged (median, 39.45% [min, 8.5%; max, 82.9%]; *P*=.104530). This stimulation was 95.45% sensitive both before and after intake of cetirizine (Figure 3).

Allergen-Induced CD63 Expression

Stimulation using D pteronyssinus: In the group of patients allergic to *D pteronyssinus* (n=16), the median percentage of CD63-positive basophils after stimulation by the higher allergen concentration was 84.15% (min, 2.4%; max, 97%). The cutoff values for positivity and specificity determined on the basis of 2-graph ROC curve analysis revealed the same value of 15% for both allergen concentrations (Figure 4). Positive

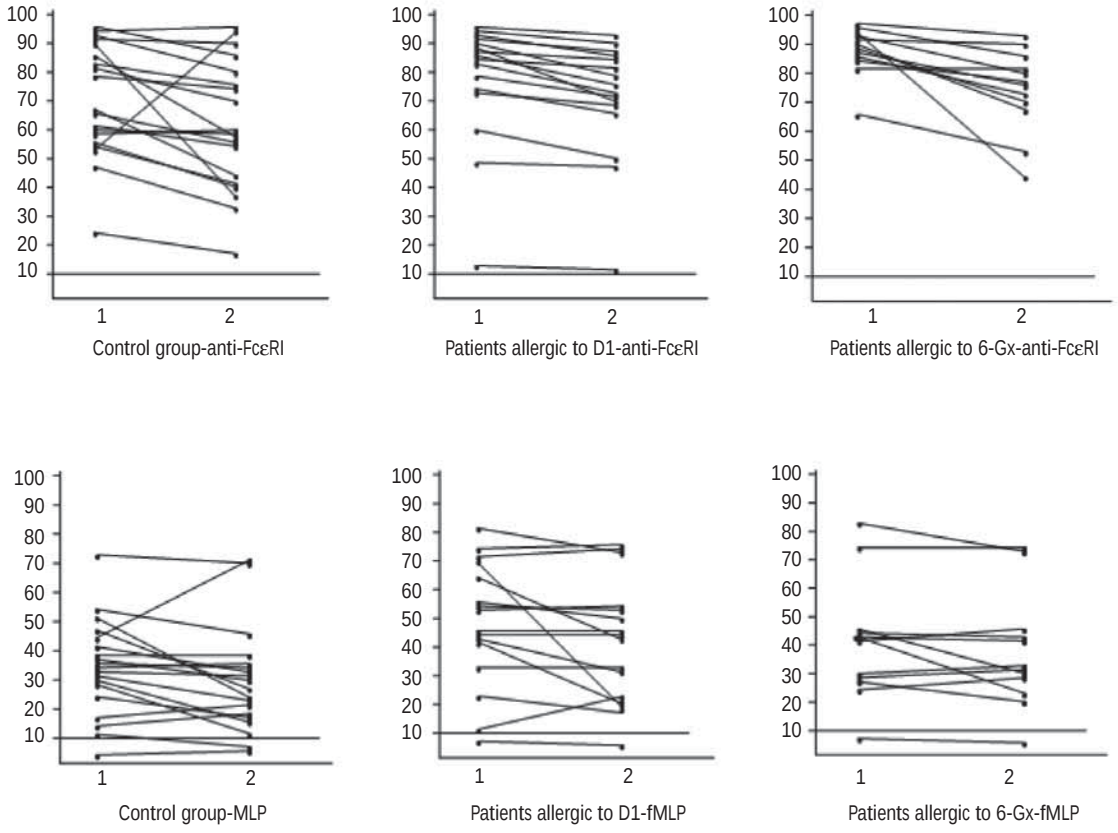

Figure 3. Positive control with anti-FcεRI antibody and formyl-Met-Leu-Phe in the control group and patients before (1) and 2 hours after (2) intake of cetirizine. fMLP indicates formyl-Met-Leu-Phe.

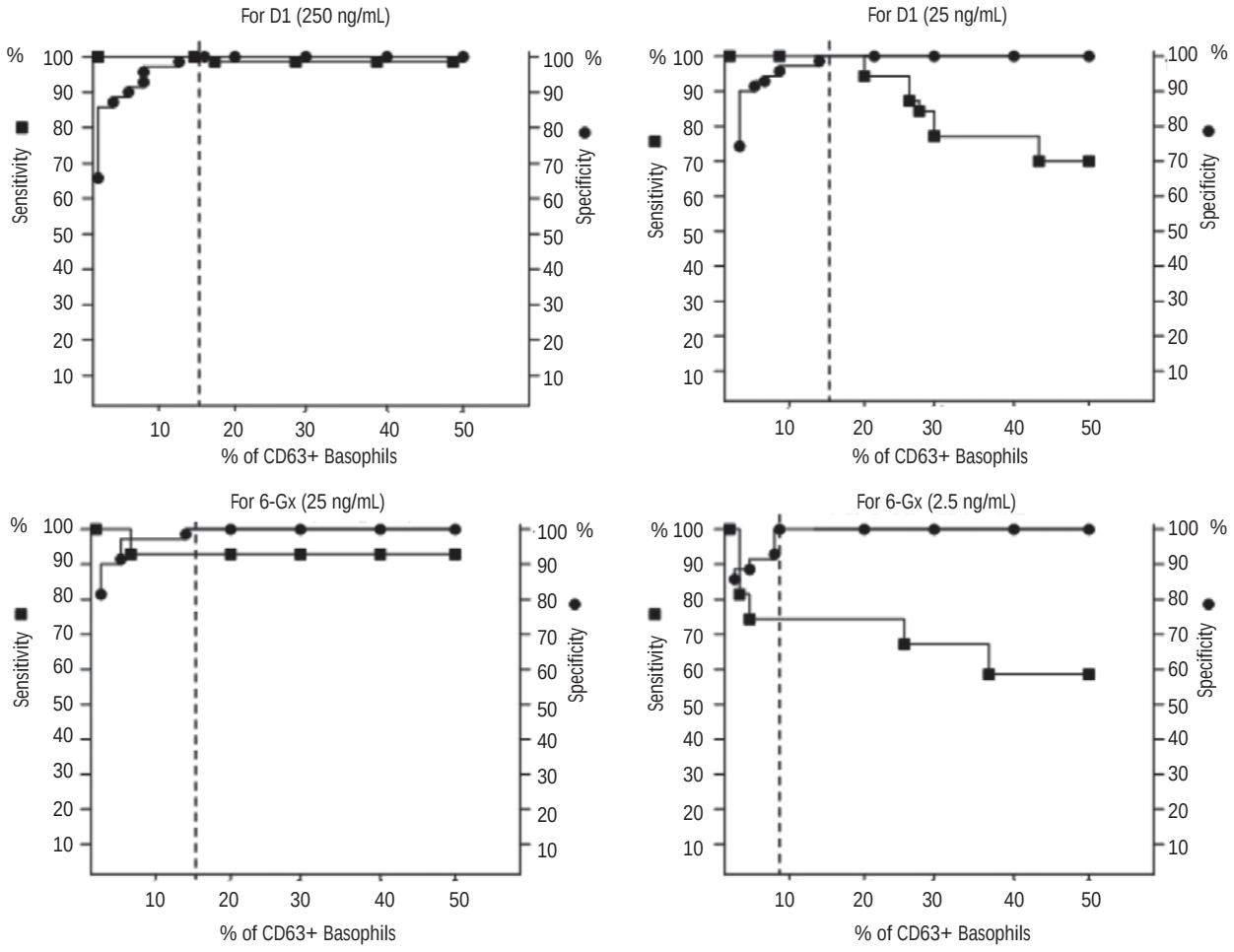

Figure 4. Determination of sensitivity and specificity of allergen-induced CD63 upregulation. Receiver operating characteristic curves for both doses of both allergens.

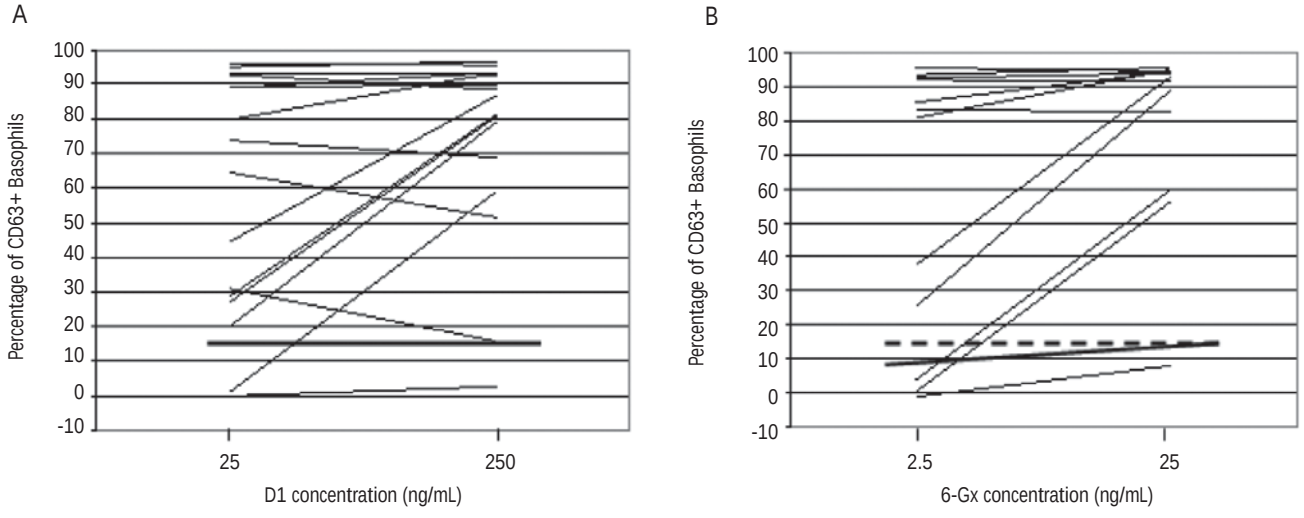

Figure 5. Dose-dependent allergen-induced CD63 upregulation in patients allergic to *Dermatophagoides pteronyssinus* (D1) (A) and to the 6-grass mix (6-Gx) (B). The solid line represents the cut points obtained in the receiver operating characteristic curve analysis and the dashed line represents the manufacturer's recommendations. The difference was found only for the lower 6-grass mix concentration.

results for allergen stimulation were observed in 15 persons (93.75% of the patients) and negative results in 1 patient (Figure 5, Figure 6, and Table). Stimulation with lower allergen concentrations resulted in a slight decrease in sensitivity. The results were positive in 14 patients (sensitivity, 87.5%) and in 2 they were negative (median, 69.25% [min, –0.3%; max, 96.3%]) (Figure 5, Figure 6, and Table 1). The dose-dependent response is presented in Figure 5.

Two hours after intake of cetirizine, the results of basophil allergen stimulation with higher concentrations of *D pteronyssinus* were significantly lower than at baseline (median, 78.2% [min, 0.9%; max, 95.9%]; *P*=.046827). The only negative result for basophil stimulation was observed in the patient who had a negative result at baseline. In the rest of the patients, basophil stimulation gave clearly positive results. In other words, test sensitivity was not influenced by cetirizine intake and remained at the same level of 93.75%. Lower concentrations of *D pteronyssinus* gave slightly weaker results than at baseline (median, 59.3% [min, -0.1%; max, 96%]; *P*=.055534). Negative results for basophil stimulation were observed in 3 patients (1 more than at baseline); consequently, sensitivity decreased from 87.5% to 81.25%. Individual results are presented in Figure 6.

In the healthy controls, basophil stimulation was negative in both allergen concentrations. The higher allergen concentration of 250 ng/mL resulted in median CD63-positive basophils at the level of 0.4% (min, –2.9%; max, 14.1%) before cetirizine intake and 0.2% (min, –1%; max, 14.2%) after cetirizine intake. The lower allergen concentration resulted in the same low level of CD63-positive basophils before cetirizine intake (median, 0.4% [min, –2%; max, 4.6%]) and after cetirizine intake (median, 0.4% [min, –0.8%; max, 14.6%]).

Stimulation using the 6-grass allergen mix: In the group of patients allergic to grass allergen (n=12), the median percentage of CD63-positive basophils after stimulation by the higher allergen concentration was 92.25% (min, 7.9%; max, 95.6%). The cutoff value for allergen stimulation positivity and specificity determined on the basis of 2-graph ROC curve analysis was 15% (Figure 4). The results for allergen stimulation were positive in 11 patients (91.66% of the patients) and negative in 1 patient (Figure 5, Figure 6, and Table). Stimulation with the lower allergen concentration resulted in a slight decrease in sensitivity (cutoff value of 9% by ROC analysis, Figure 4). The results were positive in 9 patients (sensitivity, 75%) and negative in 3 (median, 82.15% [min, –0.8%; max, 95.7%]) (Figure 5, Figure 6, and Table). The dose-dependent response is presented in Figure 5.

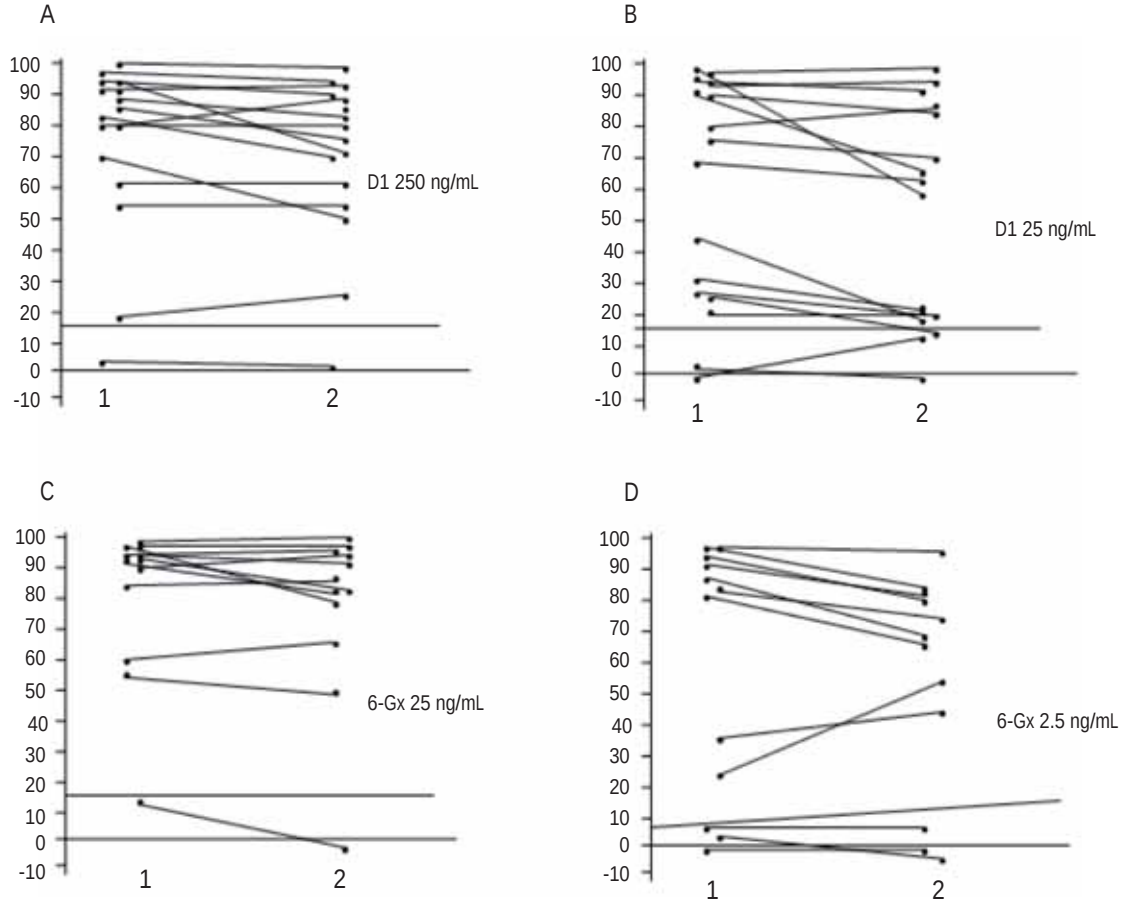

Figure 6. Basophil CD63 upregulation induced by *Dermatophagoides pteronyssinus* (D1) (A and B) and 6-Grass mix (6-Gx) (C and D) before (1) and 2 hours after (2) intake of cetirizine in the patient group.

Table. Sensitivity and Specificity of the Basophil Activation Test in Patients Who Are Allergic to *Dermatophagoides pteronyssinus* and 6-Grass Mix.

	Patients Allergic to *Dermatophagoides pteronyssinus* n=16 (19 Controls)		Patients Allergic to 6-Grass Mix n=12 (19 Controls)	
	250 ng/mL	25 ng/mL	25 ng/mL	2.5 ng/mL
Sensitivity, %	93.75	87.5	91.66	75
Specificity, %	100	100	100	100

Two hours after cetirizine intake the results of basophil allergen stimulation with the higher 6-grass allergen concentration remained unchanged (median, 82.7% [min, 0.8%; max, 96.2%] *P*=.130666). The only negative result for basophil stimulation was observed in the patient who also had a negative result at baseline. In the remaining patients, basophil stimulation gave positive results. In other words, test sensitivity was not influenced by intake of cetirizine and remained at a similar level of 91.66%. The lower concentration of the 6-grass mix also gave results comparable to baseline (median, 71.65% [min, –2.3%; max, 93.6%], *P*=.374260). The results of basophil stimulation were negative in 3 patients in whom the initial results were also negative. Therefore test sensitivity remained unchanged at 75%. Individual results are presented in Figure 6.

In the healthy control group, basophil stimulation with this allergen was negative for both allergen concentrations. The higher allergen concentration of 25 ng/mL resulted in median CD63-positive basophils at the level of 0.3% (min, –2.6%; max, 4.9%) before cetirizine intake and at the level of 0.2% (min, –1.1%; max, 14.5%) after cetirizine intake. Lower allergen concentrations resulted in the same low level of CD63-positive basophils before cetirizine intake (median, 0.2% [min, –2.6%; max, 8.1%]) and after cetirizine intake (median, 0.5% [min, 1.1%; max, 8.8%]).

Discussion

Flow cytometric BAT is a promising alternative for in vitro diagnosis of IgE-mediated reactions [23]. Most assays use CD63 upregulation based on basophil identification by anti-IgE, although some include additional antibodies to distinguish basophils from debris and other cells [6,16]. Abauf et al [14] proposed combined IgE and CD203c targeting for this purpose, and anti-CCR3 antibody has also been shown to facilitate gating of the whole, pure basophil population [20]. This simplified method of basophil gating used in tested new variants of BAT has not been validated, even in allergy to aeroallergens. The modifications presented above may lead to greater precision of basophil gating and, thus, to greater sensitivity of BAT, which could prove helpful in the diagnosis of weak in vitro reactions [3,6,15,23].

The results of studies assessing the diagnostic usefulness of new methods in the case of allergy to aeroallergens are usually compared to the gold standard, namely, skin testing. We showed that positive skin tests to allergens and histamine were strongly inhibited by cetirizine (Figure 2). These results are consistent with previous reports, which showed cetirizine-induced inhibition of skin tests, but without inhibition of the release of mast cell–derived mediators (histamine, tryptase, prostaglandin D_2) [24,25].

Values for CD63-positive basophils in unstimulated samples were very low (median, 0.9%) and only reached an exceptional value of 15.7% in 1 patient. The positive control stimulations with both the stimulators used (anti-FcεRI antibody and fMLP) gave satisfactory results, because all participants reacted to both of these agents. The higher percentage of positive results (100% vs 95.2%) and generally higher CD63 upregulation seem to indicate that anti-FcεRI antibody is more efficient than fMLP because of the higher percentage of positive results (100% vs 95.2%) and usually higher CD63 upregulation in tested individuals (Figure 3). The results of both control stimulators were statistically lower after cetirizine intake, although most participants remained strongly positive. Negativization was observed in only 1 control whose initial fMLP stimulation was relatively weak (Figure 3).

The sensitivity and specificity of the in vitro response to the relevant allergens were calculated using ROC curve analysis, and the cut point was established at 15% for both concentrations of *D pteronyssinus* and the higher concentration of 6-grass mix. This calculation confirmed the manufacturer's recommendations, although in the case of the lower concentration of 6-grass mix the cut point was 9% instead of the manufacturer's recommended 15% (Figure 4). This discrepancy does not affect sensitivity, because patients with negative results reacted below 4% and other patients with positive results reacted between 25.7% and 93.3% (Figure 5). The relevant allergens induced strong basophil activation with absolute specificity (in all controls these results were negative). A dose-dependent increase in reactivity was observed in 48% of the BAT-positive patients, inverse dependence was observed in 12% (lower results at the higher allergen concentration), and the response was almost stable in 40% (Figure 5).

Sensitivity reached 93.75% in patients allergic to *D pteronyssinus* and 91.66% in patients allergic to the 6-grass mix when the blood samples were stimulated by higher concentrations of allergens (Table). Moreover, test sensitivity was not influenced by intake of cetirizine, although a comparison of baseline results with those obtained after intake indicated a significantly lower degree of basophil activation after intake (Figure 6A and 6C). Stimulation by lower

concentrations of both allergens resulted in a slight decrease in sensitivity and almost produced resistance to cetirizine—the test results returned to negative values in 1 out of 22 patients who initially had a positive result (Figure 6B and 6D).

Although direct comparisons with other BATs have not been performed, the very high sensitivity and specificity of this test when aeroallergens are used is similar to that reported by other groups using other systems [16,18,19]. This similarity is somewhat expected, because these allergens are known as strong in vitro activators, although this simple statement does not include intensity of the response. A careful analysis of previously reported data from patients sensitized to aeroallergens reveals that the percentage of $CD63^+$ basophils was rarely higher than 80%-90% [26-29] and median (or mean) values ranged from 54.9% [16] to 73.5% [29]. In our study, the median values of $CD63^+$ basophils reached values never before observed: 92.25% (for 6-grass mix) and 84.15% (for *D pteronyssinus*). This difference indicates an extraordinarily intensive in vitro response in the system tested; however, a definitive statement should reflect direct comparisons with other systems. Also important will be comparisons of the protocol we tested with older procedures in the case of weak in vitro activators such as drugs. This issue requires several comparative studies to be made before the best protocol for different clinical needs can be selected. Our results suggest that the BAT we tested could be a promising tool, even when weaker in vitro activators are used.

One of the most important advantages of this test over previous protocols is the shorter cell stimulation time (15 minutes instead of 30 minutes) [3,10,13] and the addition of staining antibodies to the buffer at the beginning of cell stimulation (the staining step takes place during the activation step). This less time-consuming procedure enables blood specimens to be prepared in 1 hour.

In conclusion, our study showed the very high diagnostic usefulness of the new protocol for blood sampling, stimulation, and staining. The short processing time, easy basophil gating, and strong CD63 upregulation with very high sensitivity and excellent specificity make this test a promising tool in the diagnosis of allergy. Moreover, this test seems to have an important advantage over skin tests because of the very weak inhibitory effect of cetirizine on allergen-induced CD63 upregulation. Therefore, this diagnostic procedure can also be performed in patients taking cetirizine with no distinct decrease in diagnostic sensitivity. The preliminary results of our open-label study were very convincing; however, double-blind placebo-controlled trials are necessary before definite conclusions can be reached.

Acknowledgments

We are grateful Dr. Jakob Weber and Dr. Michael Schneider from Bühlmann Laboratories AG for their support in the use of the FLOW2 CAST kits.

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Manuscript received February 7, 2009; accepted for publication April 4, 2009.

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