

CD63 expression on basophils as a tool for the diagnosis of pollen-associated food allergy: sensitivity and specificity

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Summary

Background Basophil activation is associated with the expression of CD63. Because allergens can induce basophil activation by cross-linking specific IgE, increased CD63 expression has been proposed as a novel *in vitro* test for immediate type allergy.

Objective We compared the CD63-based basophil activation test (BAT) in the diagnosis of allergy to carrot, celery and hazelnut with skin prick tests (SPT) and measurement of allergen-specific IgE.

Methods Twenty-nine patients with a history of an oral allergy syndrome induced by carrot, celery or hazelnut ($n = 20$ for each allergen) and 20 controls were studied. SPT were performed with standardized and native carrot, celery and hazelnut extracts. Allergen-specific IgE was determined by the CAP FEIA method and basophil activation was determined by flow cytometry upon double staining with anti-IgE/anti-CD63 mAb.

Results SPT with native carrot, celery and hazelnut showed sensitivities of 100%, 100% and 90%, and specificities of 80%, 80% and 90%. SPT with commercial extracts of the same allergens gave sensitivities of 85%, 80% and 85%, and specificities of 80%, 80% and 90%. Sensitivity of allergen-specific IgE and the BAT for carrot, celery and hazelnut was 80% vs. 85%, 70% vs. 85%, and 80% vs. 90%, with corresponding specificities of 80% vs. 85%, 80% vs. 80%, and 95% vs. 90%. The cut-off for a positive BAT was 10% CD63⁺ basophils. Moreover, there was a positive correlation between IgE reactivity and the number of CD63⁺ basophils for all food allergens (carrot: $r = 0.69$, celery: $r = 0.67$, hazelnut: $r = 0.66$).

Conclusions Quantification of basophil activation by CD63 expression is a valuable new *in vitro* method for diagnosis of immediate type food sensitization. Although double-blind placebo-controlled food challenges remain the gold standard, the CD63-based BAT may supplement routine diagnostic tests such as SPT or allergen-specific IgE in the future.

Keywords basophil activation, basophil, CD63 expression, flow cytometry, food allergy

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Introduction

Mast cells and basophils play a dominant role as effector cells in immediate type hypersensitivity. Therefore basophils are most often used as target cells for *in vitro* assays to diagnose allergies. Activation of basophils induces fusion of cytoplasmic granules with the plasma membrane and the successive release of inflammatory mediators such as histamine [1]. This fusion phenomenon also changes the surface structure of the plasma membrane [2]. Activation of basophils can be quantified via measurement of mediators, stored or *de novo* synthesized and released in the extracellular medium [3–5]. Human basophil reactivity has been studied by examining changes in the staining patterns of the intracytoplasmic granules using toluidine, alcian or astra blue (basophil degranulation test) [6–8] or by measuring histamine release [9, 10]. Study of allergen-induced

leukotriene release (LTC₄) of basophils after pre-incubation with IL 3 has also been suggested for routine allergy testing [11]. Flow cytometry is a widely used method for analysis in clinical immunology. Human basophils, in common with other white blood cells, express many different surface markers such as high affinity IgE receptor (FcεRI), CD11, CD18, CD31, CDW32 (FcγRII), CD33, CD40, CD45, CD49d/CD29 and CD54 [2, 12–17]. Recently, it has been reported that the surface marker CD63 is expressed with high density on activated basophils yet only weakly on resting cells [12, 13, 15, 18, 19]. The CD63 glycoprotein is present on the membrane of cytoplasmic granules, is expressed only to an extent of less than 5% on the outside membrane of resting basophils, but with high density on activated basophils after the fusion of these granules with the cytoplasmic membrane [2, 20, 21]. Flowcytometric detection of basophil activation by CD63 expression has been studied in diagnosing immediate type allergy to various allergens such as natural rubber latex, foods, hymenoptera venoms, aeroallergens (cypress pollen, house dust mites) and drugs [2, 22–33]. This new *in vitro* method has been commonly referred to as basophil activation test (BAT) [22] or BASIC test (test of

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basophil altered surface immunofluorescence detected by cytometry [23]. However, validation of this basophil activation test for diagnosis of food allergy in particular is still very limited.

Therefore, the aim of this study was to investigate whether a two-colour flow cytometric method using phycoerythrin-conjugated anti-IgE and fluorescein isothiocyanate-conjugated anti-CD63 mAb to analyse human basophil activation could be applied to the diagnosis of food allergy, e.g., to carrot, celery or hazelnut, and to compare its sensitivity and specificity with other well-established tests such as skin prick tests (SPT) and measurement of allergen-specific serum IgE applying receiver operating curve (ROC) analysis.

Materials and methods

Patients

Twenty-nine patients with a most suggestive history of an oral allergy syndrome (OAS) [34] induced by carrot, celery or hazelnut (selection of 20 patients for each food) were recruited for this study. Only patients with a highly suggestive history analogous to class A of the classification by Nyfeler and Pichler to evaluate reliability of clinical history in drug allergy [35] were considered. All of the patients had a history of seasonal allergic rhinitis or allergic asthma and most of them reported an OAS to more than one of the three foods. Twenty subjects with no history of an adverse food reaction and no seasonal allergic rhinitis or asthma were selected as controls. All subjects provided informed consent before enrolment in this study.

Skin testing

SPT were performed on the flexor aspect of the forearm with a standardized prick needle (ALK Scherax, Hamburg, Germany). We used histamine dihydrochloride (10 mg/mL) as a positive control, and the glycerol diluent of the prick solution as a negative control. Patients and controls were skin prick tested with commercially available allergen extracts of carrot, celery and hazelnut (ALK Scherax) and native carrot, celery root and hazelnut by using the prick-to-prick technique [36]. Each food (allergen extract and native food) was tested in 20 patients with a suggestive history of allergy to this food and in 20 controls, giving a total of 120 skin tests in the 29 patients with suggestive food allergy and 120 tests in the controls. In addition, all subjects were skin prick tested with birch, grass and mugwort pollen (ALK Scherax). We recorded reactions after 15 min.

A skin test was considered positive if it produced a weal with a diameter of at least 3 mm [37].

Testing for IgE

Total IgE and specific IgE to carrot, celery, and hazelnut were measured by using the CAP FEIA system according to the recommendations of the manufacturer (Pharmacia & Upjohn, Uppsala, Sweden). Specific IgE was recorded in CAP classes 0 to 6 (class 0: < 0.35 kU/L, class 1: 0.35 to 0.70 kU/L, class 2: 0.70 to 3.50 kU/L, class 3: 3.50 to 17.50 kU/L, class 4: 17.50 to 50.00 kU/L, class 5: 50.00 to 100.00 kU/L, class 6: > 100 kU/L). Specific IgE exceeding 0.35 kU/L was considered positive.

Basophil CD63 surface expression

Peripheral blood samples from all subjects were drawn into heparinized tubes; 100 µL of whole blood was pre-incubated with 20 µL of wash buffer (containing IL-3 at a final concentration of 2 ng/mL) at 37 °C for 10 min; 100 µL of the cell suspension was distributed in microtitre plate wells and mixed in parallel with 100 µL of wash buffer (negative control), 100 µL of N-formyl-methionyl-leucyl-phenylalanine (fMLP) (1 µmol/L as positive control) and 100 µL of the three tested commercially available carrot, celery, and hazelnut extracts (ALK-Scherax) diluted 1 : 10. Before this study the basophil activation test had been performed in a pilot study in food allergics (seven per food item) with carrot, celery and hazelnut allergen extracts of seven increasing dilutions (1 : 2, 1 : 5, 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5}). A bell-shaped dose-response curve for all three allergen extracts was demonstrated (Fig. 1). Allergen extracts of carrot, celery and hazelnut diluted 1 : 10 yielded best results with regard to mean basophil activation. No basophil activation could be induced in the controls.

Plates were incubated for 20 min at 37 °C. Degranulation was stopped by chilling on ice for 5 min. Thereafter phycoerythrin-conjugated anti-IgE (PE anti-IgE) and FITC-conjugated anti-CD63 mAb were added and incubated for 20 min on ice. Finally, the heparinized whole blood samples were lysed, fixed and washed. Plates were centrifuged (250 g, 5 min, 4 °C) and analysed within 2 h on a FACScan flow cytometer (Becton Dickinson Immunocytometry System, San Jose, USA). IL-3, fMLP, PE anti-IgE and FITC-conjugated anti-CD63 mAb were purchased from Orpegen Pharma, Heidelberg, Germany. The basophil population was gated by the expression of PE anti-IgE. The expression of CD63 was analysed on this gated cell population. At least 500 IgE-expressing basophils per probe were acquired and results were given as percentage of basophils

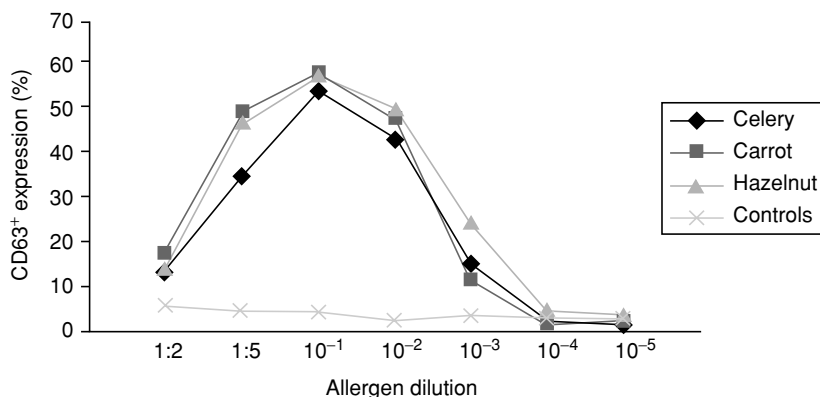


Fig. 1. CD63 expression of basophils in a dose-response curve after incubation with carrot, celery and hazelnut allergen extracts (dilution: 1:2, 1:5, 10^{-1} – 10^{-5}) in allergics ($n = 7$ for each food) and controls ($n = 9$).

expressing CD63 according to the following formula: number of PE anti-IgE⁺ and FITC anti-CD63⁺ basophils/number of PE anti-IgE⁺ basophils.

Statistics

Data are expressed as means and standard deviation. Comparison of percentage of CD63⁺ basophils between patients and controls was performed with Student's *t*-test. Significance level was set to 0.05. Because of the explorative capacity of *P*-values no α -adjustment was made.

Receiver operating characteristics (ROC) curves are used to assess the optimal cut-off (percentage of CD63⁺ basophils) for a positive basophil activation test. A ROC curve is a plot of a test's sensitivity (on the *y*-axis) versus its false positive rate (that is, 1-specificity, plotted on the *x*-axis) for different decision thresholds for defining positive and negative results [38]. Sensitivity and specificity were calculated according to the method of Goldman [39] as follows: in this study a true-positive (TP) result was defined as a patient with a positive history of an oral allergy syndrome and a positive skin prick test, or CAP or basophil activation test; a false-positive (FP) result was defined as a patient with a negative history of an oral allergy syndrome and a positive skin prick test, or CAP, or basophil activation test; a true-negative (TN) result was defined as a patient with a negative history of an OAS and a negative skin prick test, or CAP or basophil activation test; a false-negative (FN) result was defined as a patient with a positive history of an OAS and a negative skin prick test, or CAP or basophil activation test.

Sensitivity = TP/(TP + FN)

Specificity = TN/(TN + FP)

All statistical analyses were performed with MedCalc version 6.01.

Results

Skin testing

Results of 60 SPT with commercially available allergen extracts of carrot, celery and hazelnut and 60 prick-to-prick tests with native carrot, celery and hazelnut in 29 patients with a history of an OAS induced by these foods are summarized in Table 1. In the 20 controls (Table 1) only two subjects showed positive SPT to hazelnut (both to the allergen extract and the native food) and four gave positive SPT with carrot and celery (both the allergen extract and the native food induced reactions). Prick-to-prick tests with native carrot, celery and hazelnut showed sensitivities of 100% (95% CI: 83 to 100%), 100% (95% CI: 83 to 100%), and 90% (95% CI: 68 to 99%), respectively. The corresponding specificities were 80% (95% CI: 56 to 94%), 80% (95% CI: 56 to 94%) and 90% (95% CI: 68 to 99%), respectively. SPT with commercially available allergen extracts of carrot, celery and hazelnut gave sensitivities of 85% (95% CI: 62 to 97%), 80% (95% CI: 56 to 94%) and 85% (95% CI: 62 to 97%), respectively. The corresponding specificities were 80% (95% CI: 56 to 94%), 80% (95% CI: 56 to 94%) and 90% (95% CI: 68 to 99%), respectively.

In the group of patients with a history of food allergy, SPT with birch pollen was positive in 24/29 (83%) tests, with grass pollen in 17/29 (59%) and with mugwort pollen in 18/29 (62%) tests. In the controls SPT with birch pollen was positive in 3/20

(15%) tests, with grass pollen in 5/20 (25%) tests and with mugwort pollen in 4/20 (20%) tests. The pollen-sensitive subjects also showed positive SPT to carrot, celery and hazelnut. As none of the controls reported a food or an inhalant allergy, sensitization to aeroallergens and foods reflects (clinically not relevant) sensitization or latent allergy.

Testing for IgE

In the group of 29 subjects with a history of an OAS, mean total IgE was 227.88 kU/L (standard deviation (SD): 307.60 kU/L, range: 13 to 1391 kU/L), and in the 20 controls mean total IgE was 91.75 kU/L (SD: 184.51 kU/L, range: 13 to 862 kU/L).

Table 1 summarizes the results of allergen-specific IgE determination (CAP classes 0 to 6). In the 20 controls, in four subjects we found allergen-specific IgE against carrot (CAP class 1: two subjects, CAP class 2: one subject, CAP class 3: one subject) and against celery (CAP class 1: three subjects, CAP class 2: one subject). One subject showed specific IgE against hazelnut (CAP class: 3). As none of the controls reported a food allergy, specific IgE reflects clinically not relevant cross-sensitization based on history, as these patients were also sensitized to aeroallergens (positive SPT with birch, grass and mugwort pollen). Determination of allergen-specific IgE against carrot, celery and hazelnut produced a sensitivity of 80%, 70% and 80%, respectively. The corresponding specificities were 80%, 80% and 95%.

Basophil CD63 surface expression

In the 29 patients with a history of an OAS the mean percentage of CD63⁺ basophils (of the total of all IgE⁺ basophils) after incubating the basophils with wash buffer (negative control) was 2.7% (SD: 1.84%) as compared with an average activation of 2.99% of all basophils (SD: 1.56%) in the 20 controls.

Incubating the basophils with fMLP (positive control) resulted in a mean basophil activation of 30.96% (SD: 16.08%) in the food allergies and a mean basophil activation of 37.6% in the controls (SD: 16.35%).

In the group of food allergies (*n* = 20 for each food), incubating the basophils with the relevant food extract – carrot, celery and hazelnut – gave the following results with regard to mean basophil activation: carrot 48.74% (SD: 31.72%), celery 43.43% (SD: 32.33%), and hazelnut 53.92% (SD: 28.65%). Figure 2 shows basophil activation induced by a hazelnut allergen extract in one subject allergic to hazelnut. In the 20 controls mean basophil activation after incubating with carrot, celery and hazelnut was 8.55% (SD: 14.03%), 10.69% (SD: 17.66%), and 8.44% (SD: 14.99%). Comparison of percentage of CD63⁺ basophils shows significant differences between patients and controls in all three allergens. The corresponding *P*-value of Student's test was < 0.0001 for carrot, 0.0003 for celery, and < 0.0001 for hazelnut. For negative and positive controls percentage of CD63⁺ basophils did not differ between patients and controls (*P* = 0.5673 and *P* = 0.1652, respectively). Data are shown in Fig. 3.

Spearman's correlation coefficients indicated a moderate positive correlation between CAP class and CD63⁺ basophils for all food extracts (*r*_{carrot}: 0.6854, *r*_{celery}: 0.6705, *r*_{hazelnut}: 0.6557).

Applying ROC analysis to determine a best cut-off with regard to highest sensitivity and specificity gives a positivity

Table 1. Results of the basophil activation test (cut-off for a positive test: 10% CD63⁺ basophils; percentage of CD63⁺ basophils), measurement of specific IgE (CAP classes 0 to 6), and skin prick tests (SPT) with carrot, celery and hazelnut (native food and commercially available food extract) in 20 patients (no. 1 to 20) with an oral allergy syndrome induced by each food and 20 controls (nos 21 to 40)

Carrot							Celery					Hazelnut				
Basophil activation test				SPT			Basophil activation test			SPT		Basophil activation test			SPT	
OAS	+/-	%CD63 ⁺	CAP class	Native	Commercial	+/-	%CD63 ⁺	CAP class	Native	Commercial	+/-	%CD63 ⁺	CAP class	Native	Commercial	
1	+	-	3.26	0	+	-	-	4.55	0	+	+	+	49.01	2	+	+
2	+	+	76.33	3	+	+	+	44.72	2	+	-	-	0.3	0	+	+
3	+	+	33.22	1	+	+	+	22.11	2	+	+	+	82.3	2	+	-
4	+	+	57.42	6	+	+	+	94.96	2	+	+	+	55.04	3	+	+
5	+	+	94.97	2	+	+	-	3.64	2	+	+	+	20.93	2	-	+
6	+	-	2.82	2	+	+	+	42.36	3	+	-	+	92.8	2	+	+
7	+	+	51.42	2	+	+	+	26.28	2	+	+	+	40.79	2	-	+
8	+	+	32.24	2	+	+	+	77.5	2	+	+	-	5.79	2	+	+
9	+	+	91.63	2	+	-	+	33.53	2	+	+	+	45.72	0	+	+
10	+	+	40.74	2	+	+	-	3.17	0	+	+	+	43.25	3	+	+
11	+	+	47.55	0	+	+	+	45	0	+	-	+	82.69	2	+	-
12	+	+	73.97	0	+	-	+	74.61	0	+	+	+	81.6	1	+	+
13	+	+	12.66	3	+	+	+	17.84	3	+	+	+	64.86	3	+	-
14	+	-	8.91	2	+	+	+	14.7	0	+	+	+	91.68	1	+	+
15	+	+	94	2	+	+	+	95.81	3	+	+	+	25.47	2	+	+
16	+	+	29.4	0	+	+	+	30.47	0	+	+	+	40.73	0	+	+
17	+	+	11.91	3	+	+	+	12.14	2	+	+	+	28.9	2	+	+
18	+	+	54.4	3	+	+	+	48.09	3	+	+	+	70.08	2	+	+
19	+	+	65.29	2	+	+	+	84.7	1	+	-	+	92.69	0	+	+
20	+	+	92.69	3	+	+	+	92.51	2	+	+	+	63.3	3	+	+
21	-	+	27.25	1	+	+	+	14.13	1	+	+	-	49.74	0	+	+
22	-	+	61.45	3	+	+	+	57.39	2	+	-	-	4.67	0	-	-
23	-	-	4.81	0	-	-	-	6.34	0	-	-	-	6.46	0	-	-
24	-	-	3	0	-	-	-	2.6	0	-	-	-	2.65	0	-	-
25	-	-	2.18	0	-	-	-	0.36	0	-	-	-	5.83	0	-	-
26	-	-	5.3	0	-	-	-	5.5	0	-	-	-	4.6	0	-	-
27	-	-	2	0	-	-	-	1.22	0	-	-	-	1.18	0	-	-
28	-	-	3.04	0	-	-	-	3.17	0	-	-	-	2.12	0	-	-
29	-	-	7.02	0	-	-	-	2.65	0	-	-	-	3.04	0	-	-
30	-	-	2	0	-	-	-	3.9	0	-	-	-	0.38	0	-	-
31	-	-	2.3	0	-	-	-	3.7	0	-	-	-	3.5	0	-	-
32	-	-	2.5	0	-	-	-	3.5	0	-	-	-	1.3	0	-	-
33	-	-	2.2	0	-	-	-	5.8	0	-	-	-	1.51	0	-	-
34	-	-	1.8	0	-	-	-	1.75	0	-	-	-	2.21	0	-	-
35	-	-	3.53	0	-	-	-	2.58	0	-	-	-	4.91	0	-	-
36	-	-	8.6	0	-	-	-	6.1	0	-	+	-	6.7	0	-	-
37	-	+	19.6	2	+	+	+	63.02	1	+	+	+	54	3	+	+
38	-	-	6.6	1	+	+	+	20.7	1	+	-	-	5.6	0	-	-
39	-	-	3.09	0	-	-	-	3.63	0	-	-	-	2.74	0	-	-
40	-	-	2.63	0	-	-	-	3.28	0	-	-	-	5.82	0	-	-

threshold of 8.9% CD63⁺ basophils for carrot with 85% (95% CI: 62 to 97%) sensitivity and 85% (95% CI: 62 to 97%) specificity. In detecting celery allergy the best cut-off is 6.3% CD63⁺ basophils with a sensitivity and specificity of 85% (95% CI: 62 to 97%) and 80% (95% CI: 56 to 94%), respectively. For hazelnut allergy the best cut-off was 6.7% CD63⁺ basophils giving a sensitivity and specificity of 85% (95% CI: 62 to 97%) and 80% (95% CI: 56 to 94%), respectively. On the basis of our data the next positivity thresholds for carrot, celery and hazelnut are 11.9%, 12.1% and 20.9% CD63⁺ basophils, respectively, with sensitivities of 80% (95% CI: 62 to 97%), 80% (95% CI: 62 to

97%) and 85% (95% CI: 62 to 97%) and specificities of 85% (95% CI: 62 to 97%), 80% (95% CI: 62 to 97%) and 90% (95% CI: 68 to 99%), respectively. Hence, we decided to choose a positivity threshold of more than 10% activated basophils as the 'best' cut-off, as one single cut-off for all three foods combines simplified handling with an acceptable sensitivity and specificity.

In the controls a positive basophil activation test means a 'false positive' result with regard to a negative history of food allergy as 'gold standard' and criterion for selection of these subjects and reduces specificity. However, as these controls with a positive basophil activation test also had a positive SPT and

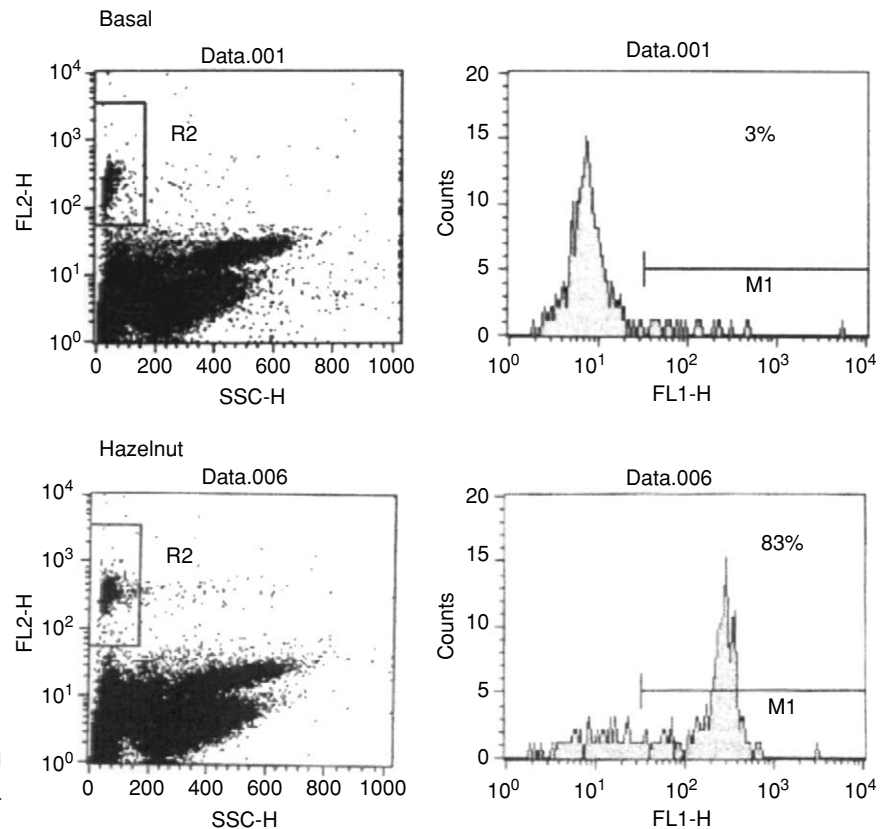


Fig. 2. CD63 expression of basophils in the basophil activation test in a patient with an oral allergy syndrome induced by hazelnut: basal (negative control without stimulation) and after challenge with a hazelnut allergen extract.

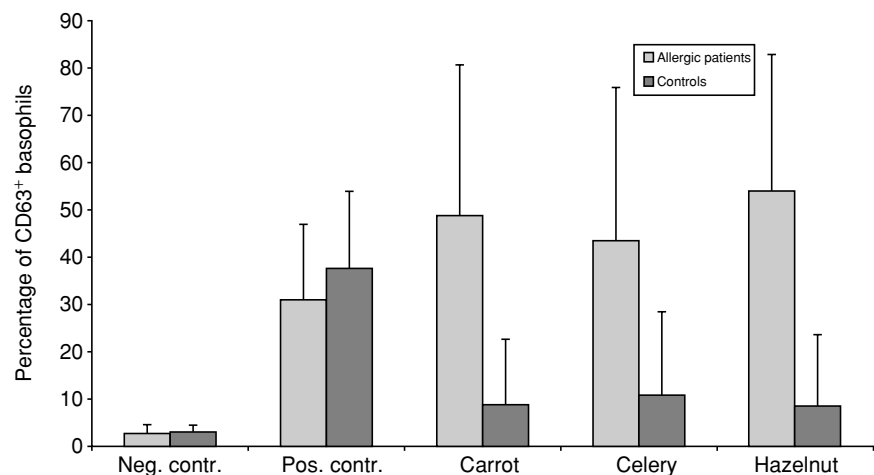


Fig. 3. Mean basophil activation (percentage of CD63⁺ basophils) and standard deviation in patients with an oral allergy syndrome induced by carrot, celery and hazelnut ($n = 20$ for each food) and 20 controls after challenge with wash buffer (negative control), fMLP (positive control) and carrot, celery and hazelnut allergen extract.

proof of allergen-specific IgE basophil activation reflects (clinically not relevant) sensitization.

Discussion

In this study flow cytometry was applied to study allergen-induced basophil activation in IgE-mediated food allergy using a double staining method with anti-IgE and anti-CD63 mAb. For studying IgE-mediated food allergy we selected patients with a pollen-associated OAS induced by carrot, celery and hazelnut. We selected these foods because recruitment of

food-allergic patients was facilitated due to the high prevalence of OAS in patients with seasonal respiratory allergy to birch pollen in particular [40, 41], ensuring a large study population for measurement of sensitivity and specificity. Identification of basophils was based on the presence of IgE on the cell surface. For study of food allergy we selected only patients with a highly suggestive history of an OAS because in former studies [42–44] that included double-blind placebo-controlled food challenge (DBPCFC) to confirm food allergy there was a high agreement between history and results of food challenges in contrast with patients showing other signs and symptoms of food allergy.

In the 29 patients with an OAS induced by carrot, celery or hazelnut, sensitivity of SPT with commercially available food extracts ranged from 80 to 85%. Testing native foods with the prick-to-prick technique increased sensitivity (raw carrot: 100%, raw celery: 100%, raw hazelnut: 90%) compared with skin testing commercially available allergen extracts. Higher sensitivity of fresh foods than commercially available food extracts in SPT is in keeping with previous studies that demonstrated an unsatisfactory allergenic potency due to unstable allergens for commercially available allergen extracts of fruits and vegetables in particular [45]. The efficiency of SPT with commercially available foods varies widely depending on the type of food tested, with poor sensitivity of apple in particular and better sensitivity of celery allergen extracts [45].

Sensitivity of SPT and determination of specific IgE by the CAP-FEIA method for carrot, celery and hazelnut in our study is consistent with results of previous studies [42–44]. In a multicentre study on hazelnut allergy, published recently, sensitivity of specific IgE determination by the CAP-FEIA method was 75%, and sensitivity of SPT with commercially available hazelnut extract was 89% [42]. Furthermore, in a recent study on food allergy to celery by Ballmer-Weber and coworkers sensitivity of determination of allergen-specific IgE (CAP-FEIA method) to celery was 73% and of SPT 48 to 86% of different commercially available food extracts and 96% of crude celery [43]. In both studies diagnosis of food allergy was confirmed by DBPCFC. Ballmer-Weber and coworkers confirmed celery allergy by DBPCFC or open oral challenge in 83% of all patients with a history of an adverse reaction to this food [43], and Ortolani and coworkers proofed hazelnut allergy in 81% of all subjects enrolled in this study [42].

In 29 patients with a suggestive history of an OAS induced by carrot, celery and hazelnut we determined CD63 expression after incubating the patients' basophils with the relevant food allergen in 20 tests for each food. An increase of CD63 expression exceeding more than 10% of the total (IgE⁺) basophil population (cut-off) was considered to be a positive basophil activation test. On the basis of this positivity threshold, 52 out of 60 tests were positive with the relevant food allergen. On the other hand, in 20 controls nine out of 60 tests (20 tests with each food) were also positive. As these controls showing a positive basophil activation test also had a positive SPT with the same food and proof of allergen-specific IgE the basophil activation was 'false positive' with regard to a lacking history of food allergy. However, in these cases a positive basophil activation test correctly identified sensitization. These data suggest that the basophil activation test, like SPT or allergen-specific IgE, does not provide definitive information with regard to the clinical relevance of sensitization.

'False positive' test results (i.e. clinically non-relevant sensitization) due to multiple cross-reactive allergens is a well-known problem in pollen-associated food allergy in particular [46]. In celery allergy IgE cross-reactivity is due to at least three distinct cross-reacting allergens [47]. Besides Bet v 1 and profilin, particularly carbohydrate structures (cross-reactive carbohydrate determinants CCD) on glycoproteins are also involved in pollen-vegetable food cross-reactivity. Their clinical relevance is, however, doubtful [48]. CCD are particularly relevant with regard to false positive IgE serology [49]. In case of monomeric binding of IgE by particular carbohydrates, cross-linking of IgE and basophil activation will not occur.

Our results confirm that detection of basophil activation by double staining with anti-CD63 and anti-IgE is a new method feasible in diagnosis of IgE-mediated immediate type allergy that widens the spectrum of *in vitro* diagnostics. These results are consistent with the outcome of several previous studies that also evaluated basophil activation by applying flow cytometry and different cell surface markers, CD63 in particular [2, 22–31]. Flow cytometric detection of basophil activation by CD63 expression has been studied in diagnosing immediate type allergy to various allergens such as natural rubber latex, foods, hymenoptera venoms, aeroallergens (cypress pollen, house dust mites), betalactam antibiotics and muscle relaxants [2, 22–31, 33]. Moreover, in several studies it has been demonstrated that CD63 expression paralleled histamine release [2, 23]. To our knowledge expression of CD63 in a large group of patients ($n = 27$) with IgE-mediated food allergy has only been investigated once before by Moneret-Vautrin and coworkers [22]. They also applied a double staining technique (anti-CD63 and anti-IgE mAb) and compared this method with determination of LTC4 and histamine release. They found detection of basophil CD63 surface expression and LTC4 release to be more sensitive than histamine release in diagnosing food allergy.

In our study sensitivity of the flow cytometric detection of basophil CD63 surface expression for carrot, celery and hazelnut was 85%, 85% and 90%, respectively. These figures were calculated on the basis of a positivity threshold of more than 10% activated basophils of the total basophil population as the minimum level for a positive basophil activation test. The corresponding specificities were 85%, 80% and 90%. Although ROC analysis implied marginally different cut-offs for a positive basophil activation test in the three foods tested (percentage of CD63⁺ basophils for carrot 8.9%, celery 6.3%, hazelnut 6.7%) we selected a positivity threshold of more than 10% activated basophils as 'best' threshold level and 'compromise' as it combines a simplified handling (same cut-off for three different food allergens) with an acceptable sensitivity and specificity.

Interestingly, determination of allergen-specific IgE by the well-established CAP-FEIA method was less sensitive than the basophil activation test in detecting sensitization to the three foods investigated. In our study applying the basophil activation test in conjunction with measurement of allergen-specific IgE, combined sensitivity of *in vitro* diagnosis in carrot allergy was 95% (sensitivity of specific IgE: 80%), in celery allergy 90% (sensitivity of specific IgE: 70%) and in hazelnut allergy 95% (sensitivity of specific IgE: 80%).

Our results suggest that the basophil activation test is an *in vitro* method feasible in diagnosis of food allergy. The sensitivity of this new *in vitro* method is comparable with the sensitivity of *in vivo* skin testing with native foods and therefore the basophil activation test may supplement routine diagnostic tests such as SPT and determination of allergen-specific serum IgE.

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