

ORIGINAL ARTICLE

Basophil activation tests for the diagnosis of food allergy in children

A. Ocmant*, S. Mulier†, L. Hanssens†, M. Goldman*, G. Casimir†, F. Mascart*‡ and L. Schandené*

*Clinique d'Immunobiologie, Hôpital Erasme, Université Libre de Bruxelles (ULB), Brussels, Belgium, †Département de Pneumo-allergologie, Hôpital Universitaire des Enfants Reine Fabiola, Brussels, Belgium and ‡Laboratoire de Vaccinologie et d'Immunologie Mucosale, Université Libre de Bruxelles (ULB), Brussels, Belgium

Clinical &
Experimental
Allergy

Summary

Background Positive skin prick tests (SPT) for food allergens and specific IgE (sIgE) in serum indicate sensitization but do not enable distinction between sensitized but tolerant and clinically allergic patients.

Objective Herein, we evaluate the clinical relevance of basophil activation tests (BATs) for peanut or egg allergy diagnosis.

Methods Thirty-two peanut-allergic, 14 peanut-sensitized (sIgE⁺ and/or SPT⁺ to peanuts) but tolerant children and 29 controls with no history of an adverse reaction to peanuts were included. Similarly, 31 egg-allergic, 14 egg-sensitized children (sIgE⁺ and/or SPT⁺ to egg white) and 22 controls were studied. Flow cytometric analysis of CD63 expression or CD203c upregulation on basophils and the production of leukotrienes (LT) were performed in response to an *in vitro* crude peanut extract or ovalbumin (OVA) challenge.

Results After *in vitro* peanut challenge, the basophils from peanut-allergic children showed significantly higher levels of activation than those from controls ($P < 0.001$). After OVA challenge, a similar distinction ($P < 0.001$) was observed between egg-allergics and controls.

Interestingly, the majority of egg- or peanut-sensitized children failed to activate basophils, respectively, in response to OVA and peanut challenge. The sensitivity of the CD63, CD203c and LT assay was 86.7%, 89.5% and 76.0% with a specificity of 94.1%, 97.1% and 94.6% for peanut allergy diagnosis. The corresponding performances of BATs applied to egg allergy diagnosis were 88.9%, 62.5% and 77.8% for the sensitivity and 100%, 96.4% and 96.4% for the specificity.

Conclusion Neither conventional tests nor BATs are sensitive and specific enough to predict food allergy accurately. However, BATs may helpfully complete conventional tests, especially SPT, allowing improved discrimination between allergic and non-allergic individuals.

Keywords basophil activation tests, diagnosis, egg, food allergy, peanut

Submitted 13 May 2008; revised 2 April 2009; accepted 6 April 2009

Correspondence:

Annick Ocmant, Hôpital Erasme,
Clinique d'Immunobiologie, Route de
Lennik 808, B-1070 Brussels, Belgium.
E-mail: aocmant@ulb.ac.be

Introduction

Egg and peanut are two of the most common offending foods that cause IgE-mediated allergies in children. Currently, a double-blind placebo-controlled food challenge (DBPCFC) [1] and open-controlled food challenges in young children are advocated as the gold standards to confirm the diagnosis of food hypersensitivity. However, these procedures are not commonly performed in practice because they have to be conducted in a hospital setting by trained personnel and require equipment to treat systemic anaphylaxis. They are time consuming and carry risks for the patient. As a result, the diagnosis of food allergy usually relies on a clinical reactivity to a particular food supported by the detection of food-specific IgE in serum

(sIgE) and positive skin prick tests (SPT). Although they are indicative of food allergen sensitization, neither of these tests is predictive of symptomatic IgE-mediated food allergy. Both in peanut and egg allergy, previous studies [2–8] correlating sIgE levels or weal diameters to oral food challenge outcomes have suggested diagnostic decision points. However, these threshold values remain indicative rather than diagnostic because they are affected by numerous factors, including age population, symptoms, technical variations. . . [9, 10]. These restrictions underline the need to develop approaches that are more reliable for diagnosing clinically reactive food allergies. Several promising tools have emerged including the component-resolved concept based on the molecular analysis of allergen sensitization patterns [11, 12] or the measurement of the patient's

basophil response to allergens, i.e. basophil activation tests (BATs). The diagnostic usefulness of *in vitro* BATs based either on the detection of allergen-induced CD63 expression or CD203c up-regulation on the basophil membrane has been demonstrated in various IgE-mediated allergies [13, 14]. However, evaluations of BATs in food allergy remain scarce [14–17] and mostly dedicated to pollen-associated food allergy syndrome [18–20]. In addition, very little information is available concerning the clinical utility of BATs for the diagnosis of food allergy in children. Several years ago, the value of histamine release test in children suffering from milk allergy [21, 22] and various food allergies [23] was documented while CD63-BAT and the leukotrienes' (LT) release was studied in children with egg and peanut allergy by Moneret-Vautrin et al. [17]. In the current study, we analyse the performance of CD63- and CD203c-BATs in the diagnosis of peanut and egg allergy in children. We also compare the flow cytometric BAT results with LT levels assessed by CAST-2000 ELISA. We demonstrate that these tests suitably complement SPT and sIgE for the diagnosis of egg and peanut allergy in children.

Materials and methods

Allergenic extracts

Defatted crude peanut powder (720401402 Allergon, Angelholm, Sweden) was extracted in phosphate buffer saline (PBS) pH 7.2 by stirring for 8 h at 4 °C and kept at –80 °C until used. Ovalbumin (OVA) (950512 ICN MP Biomedicals, Aurora, OH, USA) was chosen as the egg allergen.

Appropriate allergen dilutions were prepared in PBS pH 7.2 just before use.

Flow-cytometry-based basophil activation tests

All the tests were carried out within 4 h after blood sampling.

Allergen-induced CD63 expression was evaluated using the Basotest[®] Kit (Orpegen Pharma, Heidelberg, Germany) according to the manufacturer's instructions. Briefly, sodium heparin-anti-coagulated peripheral blood aliquots (100 µL) primed with 20 µL IL-3 containing buffer (final concentration 2 ng/mL, 10 min, 37 °C) were stimulated (20 min, 37 °C) with 100 µL of peanut (0.1–100 µg/mL), OVA (0.1–100 µg/mL) and anti-FcεRI (0.35 µg/mL, Bülhmann Laboratories AG, Schönenbuch, Switzerland) as positive control or washing solution as negative control. Thereafter, samples were mixed (20 min, on ice) with staining reagents: anti-CD63 FITC, anti-IgE PE (Basotest[®] Kit) and anti-CD45 PerCP (BD Biosciences, Erembodegem, Belgium). After erythrocyte lysis (Basotest[®] Kit), washing and centrifugation, the leucocyte pellets were re-suspended in washing solution and analysed using a six-colour flow cytometer (FACSCanto with FACSDiva software for analysis – BD Immunocytometry Systems, San Jose, CA, USA).

A minimum of 1000 basophils selected as CD45^{low}/IgE^{high} within the lymphocyte region was acquired. In the IgE/CD63 dot plots, quadrant markers were set with the negative control. The percentages of activated basophils were corrected by subtracting the spontaneous CD63 expression (quadrant 2 in negative control; see Fig. 1) from the values obtained after stimulation.

Allergen-induced CD203c up-regulation was assessed as reported previously [24] under optimal technical conditions e.g. in the absence of the IL-3 priming step. Briefly, whole blood was stimulated (37 °C, 15 min) with the same dilutions of allergens or controls used in the CD63-BAT. The reaction was stopped on ice, followed by a 20-min staining on ice with anti-IgE FITC (Caltag Laboratories, Burlingame, CA, USA), anti-CD203c PE (Coulter Immunotech, Marseille, France) and anti-CD45 PerCP (BD Biosciences). At least 1000 basophils defined as CD45^{low}/IgE^{high} in the lymphocytes gate were acquired and analysed for CD203c expression on a simple histogram (count/FL2). The results were expressed as a stimulation index (SI) calculated as the ratio of the mean fluorescence intensity of the entire basophil population obtained with and without stimulation (Fig. 1).

Allergen-specific sulphidoleukotriene release

The release of LT by allergen stimulation was measured using CAST-2000 ELISA (Bühlmann Laboratories AG, Switzerland). Within 4 h after blood sampling, whole blood taken on EDTA was sedimented on Dextran to obtain a leucocyte-enriched suspension. In the presence of IL-3, the cells were stimulated for 40 min at 37 °C with peanut or egg allergens (0.04–40 µg/mL), anti-FcεRI as positive control (0.15 µg/mL) or stimulation buffer alone as negative control. LT concentrations were measured by ELISA in the supernatants and results were expressed in picogram per millilitre.

Allergen concentrations

The dose–response curves to food allergens were analysed in six egg- and six peanut-allergic children as well as in three controls (Fig. 2). Optimal basophil response was observed at final concentrations of 10 µg/mL OVA, 1 µg/mL peanut for flow cytometric BATs and at 4 µg/mL OVA and 0.4 µg/mL peanut for CAST. The evaluation of BATs performances was performed with those concentrations.

Criteria of non-responsiveness to positive control anti-FcεRI

The non-responder status was defined as an anti FcεRI-induced CD63 expression < 10%. This represents the mean (1.99%) + 3 SD (8.04%) of CD63 expression on unstimulated basophils from all children included in this study, except four of them, who were considered as outliers because of a high baseline level of CD63 probably related to

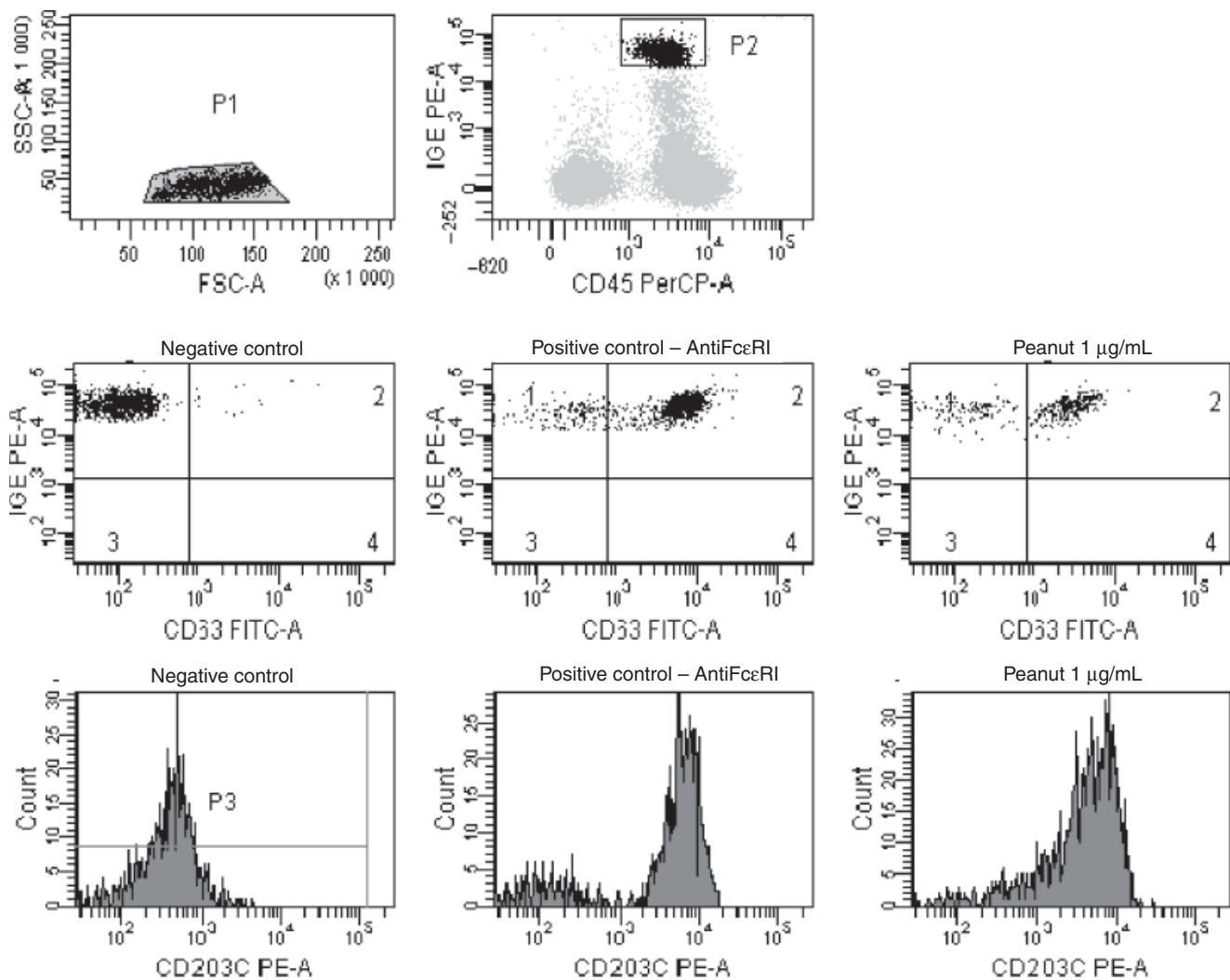


Fig. 1. Representative expression of CD63 and CD203c in basophils from a peanut-allergic patient: negative control (no stimulation), positive control (after anti-Fc ϵ RI challenge) and after allergen challenge (peanut 1 μ g/mL). Basophils are gated as described in "Materials and methods".

recent exposure to allergen. It was set at 1.2 SI for CD203c up-regulation (this corresponds to 10% CD203c⁺ basophils) and at 200 pg/mL for LT production (according to the manufacturer's instructions).

Total and specific immunoglobulin E

Total IgE and specific IgE to peanut, to the major peanut allergen Ara h 2, to egg white, to OVA, to bromelain, to profilin (Bet v 2) and to a Bet v 1 homologue (Ara h 8) were measured by the CAP-FEIA system according to the manufacturer's recommendations (Phadia, Uppsala, Sweden). Values of allergen-specific IgE below or equal to 0.35 kUa/L were considered negative.

Skin prick tests

SPT with peanut or egg white extracts (Stallergenes, Antony, France) were carried out together with positive

controls (histamine and codeine) and a negative control (saline). Skin test responses were considered positive when the weal reaction equalled or exceeded a diameter of 3 mm.

Study population

Children were attending the allergologic paediatric consultation for evaluation of suspected food allergy. According to their clinical manifestations, the children were classified as egg-allergics ($n=31$, median age 2 years [1–11]) and egg-tolerant, including both egg-sensitized ($n=14$, median age 6 years [1–12]) and controls ($n=22$, median age 5 years [1–12]). The same subdivision was adopted for peanut allergy: peanut-allergic ($n=32$, median age 5 years [1–12]) and peanut-tolerant children, including both peanut-sensitized ($n=14$, median age 3 years [1–12]) and controls ($n=29$, median age 3 years [1–11]). The diagnosis of allergy was based on at least one of the following criteria: either a clear-cut case history of

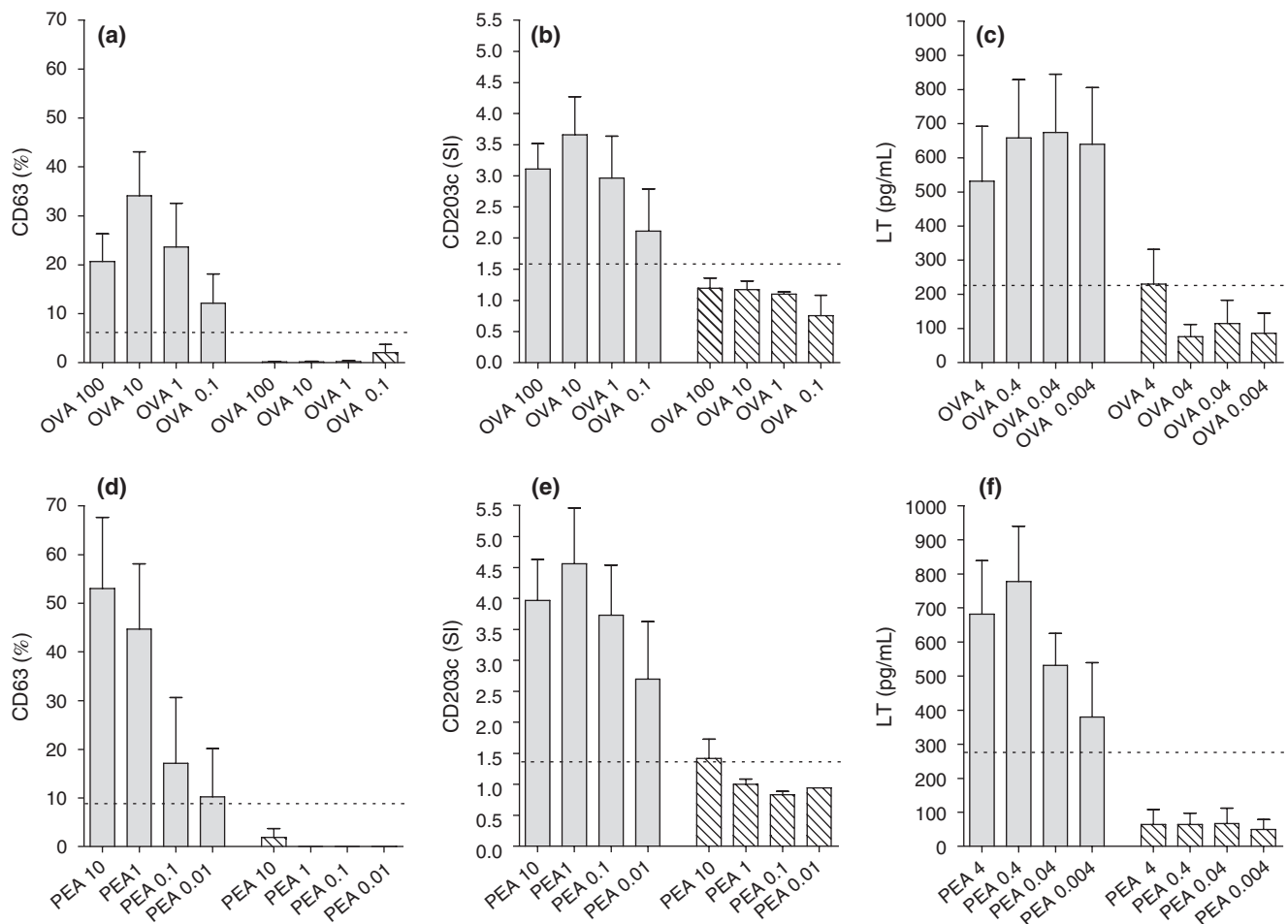


Fig. 2. Dose-response curves of CD63 (a), CD203c (b) and leukotriene production (c) obtained after stimulation of blood from egg-allergic patients ($n = 6$, on left) and controls ($n = 3$, on right) with different concentrations of ovalbumin (OVA) ($\mu\text{g/mL}$). CD63, CD203c and LT results from peanut allergies ($n = 6$) and controls ($n = 3$) depending on the peanut extract concentration ($\mu\text{g/mL}$) are presented in d, e and f.

anaphylaxis [25] in relation to the ingestion of the suspected food associated with a positive SPT and/or sIgE > 0.35 kUa/L, or a high suspicion of clinical reactivity confirmed by an oral open food challenge realized in the hospital setting under supervision with respect to a procedure described elsewhere [1, 26, 27]. Table 1 lists the clinical characteristics of peanut- and egg-allergic children. The egg-allergic population consists of 12 anaphylactic reactions, seven urticaria/angio-oedema and 12 isolated eczema. The peanut-allergic population demonstrated 22 anaphylactic reactions, one oral syndrome, three urticaria/angio-oedema and six isolated eczema. As already mentioned above, no provocation tests were performed in case of an anaphylactic reaction. Moreover, no provocation test was performed before one year of age for egg (four eczema) and before 3 years of age for peanut (six eczema and one urticaria/angio-oedema) because the investigators did not consider food challenges to be ethically acceptable. However, all these cases showed clinical improvement after eviction. Thus, a positive provocation test confirmed egg allergy in 7/7 urticaria/angio-oedema and in 7/12 eczema cases. Parents

of child 22 refused the challenge. Provocation tests to peanut were positive in 2/3 urticaria/angio-oedema and in one oral syndrome.

The sensitization status was defined as children clinically tolerant to ingestion of the relevant food but presenting either positive sIgE (7/14 for peanut and 4/14 for egg) or positive SPT (4/14 for peanut and 6/14 for egg) or both positive sIgE and SPT (3/14 for peanut and 4/14 for egg). Six out of 14 egg-sensitized patients had a resolved history of egg white reactivity. The sensitization status was based on safe consumption confirmed, if needed, by a negative oral food challenge. Controls were negative for sIgE and SPT and were consuming egg or peanut in their diets without evidence of clinical manifestations.

Statistics

All data were expressed as median [ranges] calculated on the whole population. The non-parametric one-way ANOVA/Kruskal-Wallis test was applied where appropriate. For each test, only patients responding to the positive control

Table 1. Clinical data and BAT results from (a) egg-allergic population and (b) peanut-allergic population

Patients	Age (months)	Sex	Symptoms	SPT to egg white (mm)	Egg white sIgE (kUa/L)	Ovalbumin sIgE (kUa/L)	CD63 (%)	CD203c (SI)	LTs (pg/mL)
<i>(a)</i>									
1	9	m	U, AO, V*	12	0.59	0.52	27.9	2.3	463
2	8	f	E	12	0.84	<0.35	22.8	2.8	NT
3	17	m	U, AO, E	20	0.93	0.6	<u>1.2</u>	<u>0.7</u>	0
4	23	m	AO	16	0.93	0.77	0	1.1	0
5	46	m	U, E, A*	7	1.17	0.9	<u>0.4</u>	1.4	84
6	23	f	U, AO, V, E*	14	1.44	1.33	14.5	1.5	650
7	135	f	U, AO, N**	20	1.57	1.58	28.1	2.0	5847
8	16	m	U, AO, LO*	0	1.7	1.51	80.8	NT	2118
9	12	m	U, AO, E	4	1.9	1.72	0	1.2	23
10	85	f	U, AO, A, E**	8	2.17	2.17	9.4	2.3	1664
11	52	m	U, V, E*	4	2.5	3.2	<u>0.8</u>	<u>0.9</u>	66
12	37	f	E	14	3.45	3.65	0	1.2	295
13	42	f	U, AO	14	3.66	3.51	9.2	7.5	NT
14	12	f	E	20	4.13	4.95	25.4	2.1	491
15	73	m	U, A**	25	4.89	5.26	30.4	1.6	1857
16	23	m	U, AO, D*	14	6.73	8.01	31.1	1.3	676
17	17	m	E	10	6.93	6.06	39.7	2.9	898
18	6	m	E	12	11.1	11.8	35.2	6.7	1424
19	49	f	U, AO	14	11.2	10.6	51.9	2.5	4247
20	27	m	E	12	13.8	5.37	24.6	3.3	1292
21	29	m	U, E	12	17	11.2	33.2	3.3	275
22	33	f	E	2	18.2	29.7	6.6	NT	501
23	25	m	E	14	19.1	17.2	41.7	2.9	415
24	62	f	U, AO, A**	14	21	20.4	48.4	2.8	1440
25	17	m	E	12	28.1	25.5	71.1	NT	NT
26	5	m	E	14	31.3	36.7	19.8	NT	1379
27	13	m	U, AO, LO**	5	75.3	63.3	22.0	NT	NT
28	30	m	U, AO	10	<0.35	<0.35	23.6	1.2	298
29	7	m	E	6	<0.35	0.62	<u>2.5</u>	0.8	63
30	17	f	E	10	> 100	> 100	17.2	5.0	3463
31	33	f	U, AO, A*	16	> 100	> 100	43.4	1.5	1023
Patients	Age (months)	Sex	Symptoms	SPT to peanut (mm)	Peanut sIgE (kUa/L)	Ara h2 sIgE (kUa/L)	CD63 (%)	CD203c (SI)	LTs (pg/mL)
<i>(b)</i>									
1	104	f	OS	5	0.4	<0.1	1.7	1.3	86
2	114	f	U, AO	25	0.51	0.33	14.2	2.5	123
3	16	f	E	16	0.91	1.13	57.8	NT	NT
4	42	f	U, AO	14	1.07	0.6	2.7	7.8	NT
5	9	m	E	12	1.58	1.18	34.9	2.4	327
6	48	m	U, AO, A**	2	2.4	3.89	27.3	3.4	2113
7	10	m	E	14	2.5	0.86	3.7	2.2	301
8	60	f	U, AO, LO*	25	5.5	NT	86.5	NT	NT
9	23	f	U, AO, V, E*	9	8.7	8.77	11.3	2.7	813
10	56	f	A, E*	>3	10.5	0.13	35.8	NT	1327
11	46	f	U, RC, A, E*	12	14.1	4.52	36	NT	0
12	60	f	AO, OS*	16	15.2	<0.1	85.5	7.1	1463
13	122	m	U, OS*	25	21.6	13.6	9.6	4.1	5768
14	67	f	U, AO, A**	25	34	15.8	49.4	4.7	3766
15	13	m	U, AO, E	2	46.7	1.16	21.1	NT	NT
16	92	f	U, AO, LO**	9	53.8	16.4	86.2	NT	1959
17	150	m	U, AO, LO**	NT	56.5	34.2	62.2	9.2	3182
18	41	f	AO, V, A**	25	60	69.9	90.2	NT	3424
19	85	f	U, AO, LO**	25	73.3	57	81.7	6.4	2517

Table 1. continued

Patients	Age (months)	Sex	Symptoms	SPT to peanut (mm)	Peanut sIgE (kUa/L)	Ara h2 sIgE (kUa/L)	CD63 (%)	CD203c (SI)	LTs (pg/mL)
(b)									
20	62	f	CR***	14	84.2	22.1	61.3	2.9	613
21	33	f	E	2	86.2	64.4	35.3	NT	1251
22	18	m	E	12	99.8	NT	31.4	7.2	NT
23	108	f	U, V, A**	8	149	51.8	62.8	NT	1033
24	78	f	U, AO, LO**	14	<0.35	<0.1	5.5	1.4	NT
25	70	m	U, AO, OS, E, V*	4	<0.35	0.37	10.6	NT	128
26	72	m	U, V, A**	3	<0.35	NT	<u>1.4</u>	NT	44
27	70	f	U, AO, A*	25	>100	>100	82.8	NT	318
28	120	f	U, AO, OS, V*	NT	>100	61	65.7	5.7	0
29	17	f	E	20	>100	>100	25.8	4.3	2450
30	126	m	U, AO, V, OS*	16	>100	72.5	21.1	4.9	875
31	38	m	U, AO, OS*	25	>100	>100	<u>5.0</u>	0.9	NT
32	84	f	U, AO, A**	20	>100	90.2	44.2	NT	801

Clinical data and BATs result from egg- and peanut-allergic children. Results of CD63, CD203c and CAST tests were obtained after an ovalbumin or a peanut challenge of blood from egg- and peanut-allergic children, respectively. Underlined results correspond to non-responders to the positive control anti-FcεRI.

The severity of anaphylactic reactions is classified as *grade 1, **grade 2 and ***grade 3 according to Muraro et al. [25].

U, urticaria; AO, angio-oedema; LO, laryngeal oedema; A, asthma; V, vomiting; OS, oral syndrome; E, eczema; D, diarrhoea; F, female; CR, cardiovascular reaction; M, male; N, neurological reaction; NT, not tested; BAT, basophil activation tests; SPT, skin prick test; LT, leukotrienes.

anti-FcεRI were included to construct receiver-operating characteristic curves (ROC). Analysis of ROC curves was performed between tolerant and allergic children to calculate the most accurate threshold values (minimal false-negative and false-positive results). The Spearman rank test (*r*) was used for non-parametric correlation analysis.

Ethics

All parents gave their informed consent, and the institutional ethical committees approved the protocol.

Results

Total immunoglobulin E, specific immunoglobulin E and skin prick tests results

Total IgE were higher in peanut-allergic children (670 kU/L [10–16460]) as compared with peanut-tolerant children (127 kU/L [2–3179]) ($P < 0.01$). No statistically significant differences were obtained for total IgE when other groups were compared.

With a threshold value of 0.35 kUa/L, 29/31 egg-allergic children were found to be positive for both egg white (4.13 kUa/L [<0.35 – >100]) and OVA sIgE (5.0 kUa/L [<0.35 – >100]). SPT for egg white were positive in 29/31 egg-allergic children (12 mm [<3 –25]). Eight out of 14 egg-sensitized patients displayed egg white sIgE (0.45 kUa/L [<0.35 –4.9]) and OVA sIgE (0.43 kUa/L

[<0.35 –1.98]), whereas the SPT to egg white was positive in 10 egg-sensitized children (5 mm [<3 –10]) and not available for one child (Tables 1a and 3).

Peanut sIgE were detected in 29/32 peanut-allergic children (27.8 kUa/L [<0.35 – >100]) and in 10/14 peanut-sensitized children (0.69 kUa/L [<0.35 –20.4]). Because Ara h 2 is one of the most frequently recognized peanut allergens in children [28], a retrospective analysis of IgE reactivity to Ara h 2 was performed in 29 peanut allergics and in 13 peanut-sensitized children (serum was lacking for four children). Ara h 2 sIgE was found to be positive (13.6 kUa/L [<0.35 – >100]) in 24/29 allergics and in one sensitized child (0.43 kUa/L). Low Ara h 2 values (between 0.1 and 0.35 kUa/L) were obtained in two additional peanut allergics and two sensitized subjects. The remaining three peanut-allergic subjects (Ara h 2 sIgE <0.1 kUa/L) displayed Ara h 8 sIgE (0.6, 23.3 and >100 kUa/L). SPT for peanut were positive in 28/30 peanut-allergic children (14 mm [<3 –25]) (not available in two children) and in 7/14 peanut-sensitized children (3 mm [<3 –12]) (Tables 1b and 3).

These results indicate that using the classical positivity thresholds of 3 mm and 0.35 kUa/L, SPT and sIgE offer high sensitivity but do not accurately identify those with clinically relevant sensitization (Table 2).

Basophil reactivity to positive control anti-FcεRI

Classically, some subjects are called non-responders in BAT assays because their basophils do not react to the

Table 2. Sensitivity and specificity of specific IgE, skin prick tests (SPT) and BATs considering all children (responders and non-responders to the positive control anti-FcεRI) or only responders to positive control

	Cut-offs	Calculated on responders only (%)		Calculated on the whole population (%)	
		Sensitivity	Specificity	Sensitivity	Specificity
sIgE to egg white	0.35 kUa/L			93.5	92.6
SPT to egg white	3 mm			93.5	84.6
CD63 to ovalbumin	5.0%	88.9	100	77.4	100
CD203c to ovalbumin	1.6 SI	62.5	96.4	57.7	96.6
CAST to ovalbumin	220 pg/mL	77.8	96.4	77.8	96.6
sIgE to peanut	0.35 kUa/L			90.6	76.7
SPT to peanut	3 mm			90.0	83.7
CD63 to peanut	9.1%	86.7	94.1	81.3	95.4
CD203c to peanut	1.4 SI	89.5	97.1	89.5	97.2
CAST to peanut	280 pg/mL	76.0	94.6	76.0	94.6

Cut-offs for the CD63, CD203c and LT assay are based on ROC curves.

BAT, basophil activation tests; LT, leukotriene; SI, stimulation index; ROC, receiver-operating characteristic curves.

positive control stimulation. However, until now, no clear definition of 'true' non-responders has appeared in the literature and most studies exclude from the analysis all subjects non-responding to the positive control. Based on criteria defined in "Material and methods", 17% of the children included in the present work were considered as non-responders in CD63-BAT, 3% in CD203c-BAT and none in the CAST test. None of these subjects responded to the allergen challenge. Among the responder population, the levels of activation to anti-FcεRI ranged from 12.4% to 93.5% (median = 62.6%) in CD63-BAT, from 1.3 to 31.9 SI (median = 4.3 SI) in CD203c-BAT and from 245 to > 6000 pg/mL (median = 2377 pg/mL) in the LT production assay. There were no significant differences in the anti-FcεRI-induced responses between the groups of allergic, sensitized or control subjects.

Sensitivity and specificity of the basophil activation tests

Response to the major egg white allergen, ovalbumin. The individual results of BATs after an *in vitro* OVA challenge are shown in Fig. 3. The median [range] activation levels for egg-allergic patients were 25.4% [0–80.8], 2.2 SI [0.8–7.5], 650 pg/mL [0–5847] for CD63, CD203c and LT production, respectively, compared with 0.1% [0–1.5], 1.0 SI [0.7–1.7], 4 pg/mL [0–114] for controls and 0% [0–0.9], 1.0 SI [0.7–1.3], 34 pg/mL [0–541] pg/mL for egg-sensitized children.

ROC curve analysis between egg-tolerant and egg-allergic children generated threshold values of 5.0%, 1.6 SI and 220 pg/mL for CD63, CD203c and LT protocols, respectively. A comparison of the sensitivities and the specificities of the three BATs is shown in Table 2. Of the three assays, the CD63-BAT discriminated between egg-allergic and non-allergic children with the highest sensitivity of 88.9%. As compared with routine diagnostic tests

sIgE or SPT, BATs were less sensitive, particularly when including non-responders but were more specific, achieving at least 95% specificity. Interestingly enough, no egg-tolerant individuals (including 22 controls and 14 egg-sensitized children) exhibited positive CD63-BAT. Thus, a positive CD63-BAT helpfully indicates an egg allergy diagnosis. In the present study, CD63-BAT was positive (6.6%, 23.6% and 80.8%) in all three egg-allergic patients presenting divergent results for sIgE and SPT.

Response to peanut allergen. After an *in vitro* peanut challenge, the three BAT protocols clearly discriminated peanut-allergic patients from tolerant ones (Fig. 3). The median [range] activation levels for allergic patients were 35.5% [1.7–90.2], 4.1 SI [0.9–9.2] and 875 pg/mL [0–5768] for CD63, CD203c and LT production compared with 0% [0–8.5], 1.0 SI [0–1.2] and 12 pg/mL [0–251] for controls and 0.2% [0–38.2], 1.0 SI [0.8–3.3] and 0 pg/mL [0–1738] pg/mL for peanut-sensitized children.

The cut-off values generated by ROC curves were as follows: 9.1% for CD63, 1.4 SI for CD203c and 280 pg/mL for the LT assay. As shown in Table 2, the sensitivities of the CD63- and CD203c-BATs are similar. Clearly, both CD63- and CD203c-BATs provide rather good specificities (94.1% and 97.1%), higher than SPT. Neither SPT nor BATs alone was sensitive and specific enough to support a peanut allergy diagnosis. The sensitivity was improved when CD63-BAT and SPT results were combined, peanut allergies being at least BAT⁺ ($n = 5/32$) or SPT⁺ ($n = 6/32$) and in most cases both BAT and SPT positive ($n = 21/32$). Moreover, a positive CD63-BAT, together with an SPT > 3 mm, correctly identified peanut allergy. Inversely, negative SPT, together with negative CD63-BAT, represented a reliable way to exclude peanut allergy.

Response to a non-relevant allergen. In order to assess the antigenic specificity of CD63, CD203c and LT responses to

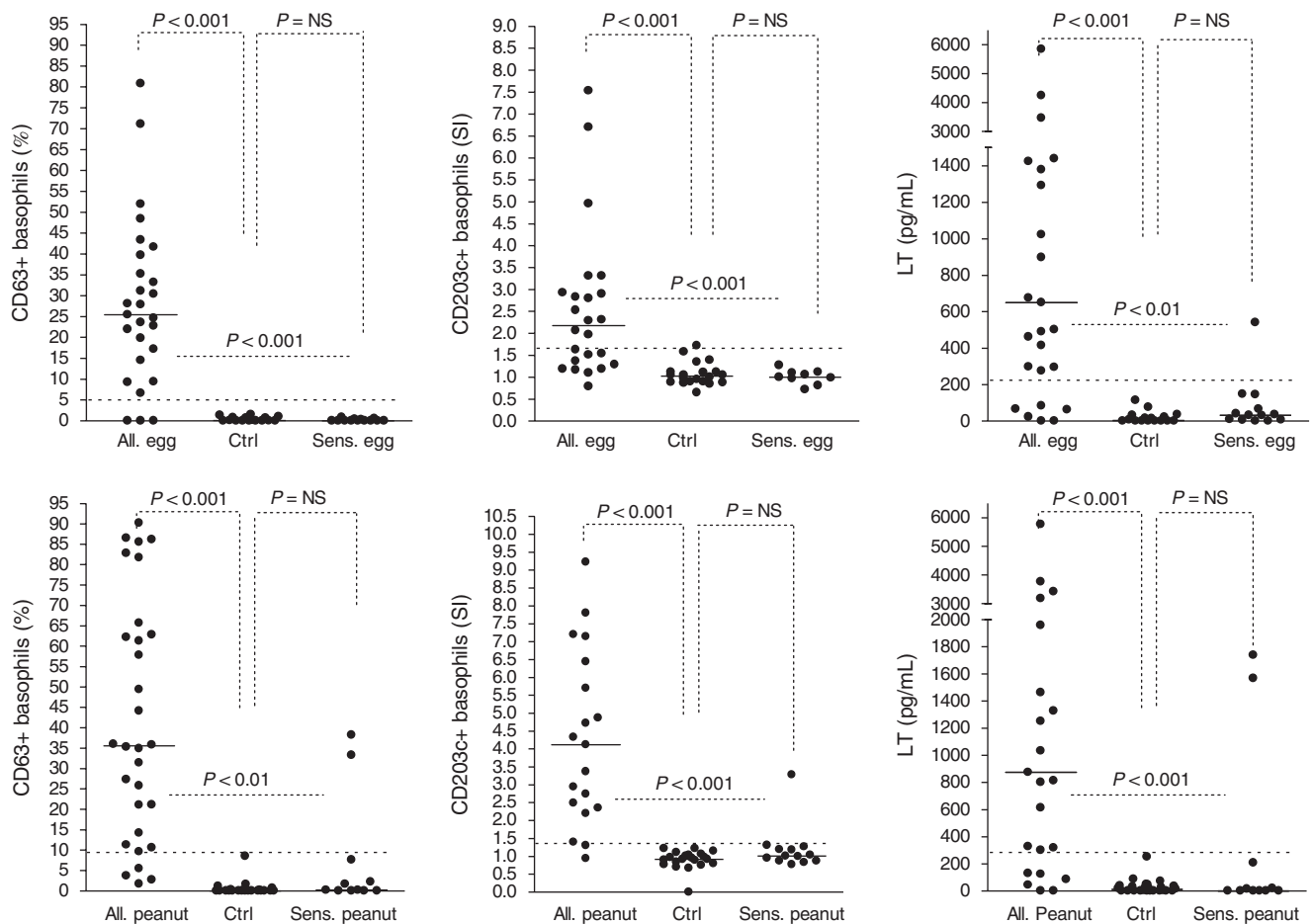


Fig. 3. Individual results of basophil activation tests. Upper panel: ovalbumin-induced CD63 expression (%), CD203c up-regulation [stimulation index (SI)] and leukotriene release (pg/mL) in egg-allergic children, controls and children sensitized to egg white. Lower panel: peanut-induced CD63 expression (%), CD203c up-regulation (SI) and leukotriene release (pg/mL) in peanut-allergic children, controls and children sensitized to peanut. Dotted lines represent the respective threshold values. Solid lines denote medians.

a relevant allergen, blood samples from six subjects allergic to peanut but tolerant to egg (negative sIgE and SPT to egg) were challenged with OVA while blood samples from six children allergic to egg but tolerant to peanut (negative sIgE and SPT to peanut allergen) were stimulated with peanut extract. Whatever the read-out considered, none of them showed a positive BAT response to the non-relevant allergen. Incubating blood samples from egg-allergic patients with a peanut extract resulted in a basophil response of 0% [0–1.63], 0.97 SI [0–1.15] and 41 pg/mL [0–251] in the CD63, CD203c and CAST test. Similarly, the basophil response of peanut-allergic patients to OVA was 0.7% [0–1.4], 0.97 SI [0.87–1.12] and 0 pg/mL [0–77].

Basophil response of individuals sensitized but tolerant to peanut or egg white

Further analysis of the results was performed to investigate whether BAT assays could be useful to predict clinical

tolerance in egg- as well as peanut-sensitized but asymptomatic children (see individual data Table 3).

Figure 3 shows that BAT results fit with the clinical response to egg white. Indeed, except for one egg-sensitized individual having a positive CAST test, no CD63 expression or CD203c up-regulation by basophils was noted after OVA stimulation. Among the peanut-sensitized children ($n = 14$ including seven sIgE⁺/SPT[−], three sIgE⁺/SPT⁺ and four sIgE[−]/SPT⁺), the results from the BAT in response to peanut allergen were significantly different from those obtained for peanut allergies ($P < 0.01$ for CD63, $P < 0.001$ for CD203c and $P < 0.001$ for LT production) (Fig. 3). The presence of false-positive peanut sIgE may arise from cross-reactivity with the so-called carbohydrate determinants (CCD) [29] but also with Bet v 1 homologues or profilin [30]. Whereas sIgE to Ara h 8 and Bet v 2 were all below 0.35 kUa/L, sIgE to bromelain were found in 4/10 individuals but these four children had negative SPT and BATs.

The analysis of the individual data indicated that only 2/14 peanut-sensitized children (sIgE⁺ but SPT[−]) had a

Table 3. Characteristics of egg- and peanut-sensitized patients

Other allergies		Total IgE (kU/L)	Egg white sIgE (kUa/L)			Ovalbumin sIgE (kUa/L)	SPT to egg white (mm)	CD63 (%)	CD203c (SI)	LTs (pg/mL)	
Data from egg-sensitized children											
1	Pollens, house dust mites, hazelnut, mustard, peanut	> 5000		1.73	1.27	10	0	1.1	148		
2	Grass pollen, house dust mites, peanut, hazelnut	1920		4.9	0.8	NT	0	NT	145		
3	House dust mites	52		<0.35	<0.35	5	0.4	0.8	0		
4	House dust mites, hazenuts, walnuts	212		<0.35	<0.35	3	0	1.3	41		
5	House dust mites, peanut	543		<0.35	<0.35	6	0	NT	9		
6	Walnut, kiwi, soy, lentils, peanut	1437		1.84	1.14	14	0	NT	541		
7	Birch pollen, peanut	1136		<0.35	<0.35	4	0	NT	7		
8	Various fruits, peanut	2109		0.4	0.4	<3	0	1	6		
9	Hazelnut, cow's milk, egg white	81		<0.35	<0.35	14	0.3	NT	NT		
10	None	91		2.44	1.52	<3	0.6	1.1	31		
11	None	8		<0.35	<0.35	5	0.9	1	66		
12	Grass pollen, house dust mites, hazelnut	476		1.62	1.98	5	<u>0.2</u>	1.1	32		
13	House dust mites	330		0.5	0.46	4	0	0.7	0		
14	Birch pollen, grass pollen	307		1	1.23	<3	0	1	36		
Other allergies		Total IgE (kU/L)	Ara h 8 sIgE (kUa/L)	Bet v 2 sIgE (kUa/L)	Bromelain sIgE (kUa/L)	Peanut sIgE (kUa/L)	Ara h 2 sIgE (kUa/L)	SPT to peanut (mm)	CD63 (%)	CD203c (SI)	LTs (pg/mL)
Data from peanut-sensitized children											
1	Cow's milk, egg white	297	<0.35	<0.35	<0.35	0.47	<0.1	<3	38.2	1.2	1738
2	Egg white	40	<0.35	<0.35	<0.35	0.69	0.32	<3	33.3	3.3	1568
3	Egg white, hazelnut	198	NT	NT	<0.35	2.03	NT	<3	<u>4.4</u>	NT	NT
4	Grass pollen, hazelnut, fish, egg white, house dust mites	3179	<0.35	<0.35	0.77	3.83	0.43	<3	2.2	1	0
5	Grass pollen, house dust mites	476	<0.35	<0.35	5.62	4.27	<0.1	<3	<u>0</u>	1	0
6	Grass pollen, birch pollen, hazelnut, Wheat	2946	<0.35	<0.35	16.1	16	0.2	<3	0	1	0
7	Lentils, fish	1947	<0.35	<0.35	9.83	20.4	<0.1	<3	0	0.8	0
8	Birch pollen, mustard, egg white	1541	<0.35	<0.35	<0.35	0.47	<0.1	3	0.2	0.9	207
9	Cow's milk, egg white, hazelnut	676	<0.35	<0.35	<0.35	0.83	<0.1	5	0	0.9	NT
10	Egg white, milk, dog, house dust mites	722	<0.35	<0.35	<0.35	1.47	<0.1	6	0	1.3	18
11	Hazelnut, cow's milk, egg white	64	NT	NT	NT	<0.35	<0.1	3	1.7	1.2	0
12	Grass pollen, moulds, house dust mites	527	NT	NT	NT	<0.35	<0.1	3	0.2	0.8	14
13	House dust mites	20	NT	NT	NT	<0.35	<0.1	10	<u>0.6</u>	1	0
14	Cat, egg white	157	NT	NT	NT	<0.35	<0.1	12	7.6	1.3	NT

Results of CD63, CD203c and CAST tests were obtained after an ovalbumin or peanut challenge of blood from egg and peanut-sensitized children, respectively. Underlined results correspond to non-responders to the positive control anti-FcεRI.

NT, not tested; SPT, skin prick test; LT, leukotriene; SI, stimulation index.

positive response to an *in vitro* peanut challenge, one for CD63 (33.3%), CD203c (3.3 SI) and CAST (1568 pg/mL) and the other for CD63 (38.2%), CAST (1738 pg/mL) but not for CD203c (1.2 SI). This observation implies that BAT

results have to be interpreted in an integrated way especially with the SPT outcome. In case of disagreement between SPT and BAT responses to peanut, a food challenge remains mandatory.

Agreement and correlation between basophil activation tests results

Several subjects were tested for all three BAT protocols: 15 peanut allergic, 25 peanut-tolerant (including 16 controls and nine peanut-sensitized), 20 egg-allergic and 19 egg-tolerant (including 11 controls and eight egg-sensitized). For the OVA allergen, concordance of the results obtained with the three BAT tests was noted in 87% of the children. Concordance between CD63 and CD203c-BAT results was observed for 90% of the children, between CD63 and CAST tests for 97%, and between CD203c and CAST results for 87% of the children. For peanut allergen, concordance was observed in 90% of children for the three tests, 95% (CD63 – CD203c), 93% (CD63 – CAST) and 93% (CD203c – CAST) (Table 4). In contrast, the correlation coefficients were rather weak, with the exception of results for peanut-induced CD203c and CD63 BAT ($r = 0.77$).

Discussion

The diagnosis of food allergy most often relies on the clinical history and on the detection of food-specific IgE either *in vivo* by SPT or *in vitro* by serum sIgE assays. The presence of specific IgE is, however, only a proof of sensitization to the corresponding antigen but does not equate with clinical allergy. For this reason, DBPCFC remains the gold standard [1] for diagnosing egg and peanut IgE-mediated allergies. Unfortunately, this procedure is only performed in specialized centres. Accurate identification of egg and peanut-allergic children is clinically quite important because reactions can be severe and undiagnosed cases may even be fatal [31]. In contrast, an incorrect diagnosis in children who are only sensitized but not allergic to these antigens is very stringent as these children will be obliged to adhere to a strict diet.

We therefore investigated whether *in vitro* basophil activation assays performed in response to the relevant food allergen could help the physicians in their diagnostic work-up. These tests are based either on quantitative determination of the LT released or on a flow cytometric assessment of CD63 membrane expression by basophils, in response to FcεRI-bound IgE cross-linking by specific allergens. The clinical benefit of such functional techniques was already demonstrated in different types of IgE-mediated food allergy [15]. However, their application for the diagnosis of egg or peanut allergy was only suggested by Monneret-Vautrin et al. [17], who compared, in a small cohort of children, the CD63 expression and concentrations of released LTs. Measurement of CD203c up-regulation on basophils also represents a useful approach in the diagnosis of allergy [24, 32] but, up to now, it has been poorly studied in food allergy [15, 16]. Thus, here we aimed to compare the performances of CD63, CD203c and LT measurement for the diagnosis of egg and peanut hypersensitivity.

Table 4. Concordance between the three BAT methods performed on the same blood sample

	CD63	CD203c	LTs	
<i>Egg</i>				
Allergics ($n = 20$)	+	+	+	13/20
	+	–	+	4/20
	–	–	–	2/20
	–	–	+	1/20
Tolerant ($n = 19$)	–	–	–	19/19
<i>Peanut</i>				
Allergics ($n = 15$)	+	+	+	11/15
	–	+	+	1/15
	+	+	–	2/15
	–	–	–	1/15
Tolerant ($n = 25$)	+	+	+	1/25
	+	–	+	1/25
	–	–	–	23/25

BAT, basophil activation tests; LT, leukotriene.

Our data indicate that among patients able to respond to FcεRI cross-linking, the flow cytometry-based BATs represent sensitive ($> 85\%$) *in vitro* tests for the diagnosis of egg or peanut allergy. A lower sensitivity (62.5%) was observed for the CD203c test when applied to the diagnosis of egg white allergy. Regarding specificities, the three methods offered excellent results, the values being between 94.1% and 100%. This is in accordance with previous studies focusing on BATs applied to various allergens [14]. Although a good concordance was observed between the flow cytometric BAT methods, it remains an open question as to whether detecting food-induced CD203c instead of CD63 or rather a combination of both markers could improve the efficiency of BATs. Further prospective multi-centre studies performed on large patient populations are required to clarify this point.

Obviously, the non-responder status [33] observed here in 17% of children represents a limitation for the use of flow-cytometry-based BATs. The true non-responders status is usually defined as non-responsiveness to anti-IgE or anti-FcεRI irrespective of the read-out. In this study, several patients were found to be negative in either CD63 and/or CD203c but never in CAST. In such cases, the diagnosis can only be based on the results from the CAST test. Nevertheless, as compared with BATs based on LT measurement, flow cytometric CD63/CD203c techniques represent faster and easier ways to detect activated basophils.

Anyway, an original issue addressed by the present work was to analyse the contribution of BATs to the distinction between allergic and sensitized children, i.e. clinically tolerant but demonstrating a positive sIgE and/or SPT. Food sensitization is quite common in early childhood [34] and represents a real diagnostic pitfall for the physician. In most cases, a given sensitization status should be further explored by an oral food challenge.

Interestingly, here we observed that most egg- or peanut-sensitized but tolerant children showed BAT responses to the putative allergen that were comparable to the non-allergic children. These results suggest that in such patients, specific IgE are unable to elicit basophil activation upon allergen challenge. This raises the question of the relationship between sensitization and clinical allergic status. Several mechanisms may be evoked like the low diversity of sIgE [35, 36], the sequential vs. conformational allergenic epitope recognition by IgE or the low avidity of IgE. Also, the presence of cross-reactive IgE directed to CCD or profilin leads to false-positive peanut-specific IgE and represents a risk for misdiagnosis especially in subjects who are polysensitized to pollens. While these antibodies are particularly frequent, they are usually claimed to be clinically irrelevant and devoid of functional activity [29]. Latex or peanut sIgE attributable to cross-reactive IgE directed to CCD of glycoprotein have been proven to be poor basophil activators [29, 37]. In the present study, CCD positivity occurs only in 4/14 peanut-sensitized but tolerant cases as confirmed by an oral food challenge. However, the occurrence of anti-CCD sIgE is not sufficient to discard the diagnosis of peanut allergy, because concomitant CCD sIgE and true peanut allergy may occur [38] and because some subjects could have biologically relevant CCD sIgE as described in the case of tomato allergy [39]. Our data showed that no CCD-positive peanut-tolerant children demonstrated basophil activation, whatever the read-out.

Taken together, it emerges that neither conventional tests nor BATs are sensitive and specific enough to predict food allergy accurately. Usually, the clinical history drives the diagnosis and SPT/sIgE, despite their moderate specificity, are indicative enough to confirm the clinical suspicion. We suggest that BATs might be useful in selected cases to characterize children showing discrepancies between SPT, sIgE and clinical history. The overall results of this study indicate that the combination of tests will offer a better diagnostic accuracy. Indeed, we found that peanut or egg allergy was always associated with at least a positive SPT or a positive BAT, that both SPT and BAT positivity provide reliable information to support allergy, while negative BAT together with negative SPT strengthens the diagnosis of peanut or egg tolerance. Oral food challenge would still be mandatory in case of divergence between SPT and BAT results and also in case of non-responders.

It is noteworthy that future improvements in the standardization of allergens or the use of recombinant peptides will greatly support the development of BATs. Moreover, as already suggested for venom immunotherapy monitoring [40], it will also be interesting to evaluate the potential of BATs for the follow-up of egg allergy over time.

In conclusion, here we demonstrate that whole blood BAT, based either on the detection of allergen-induced CD63 expression or CD203c up-regulation on the basophil

membrane, are reliable and straightforward *in vitro* methods for the diagnosis of egg or peanut allergy in children. Interestingly, BATs could provide an additional tool to discriminate between clinically relevant food-specific IgE vs. irrelevant IgE responses. The use of BATs might be justified when skin tests or specific IgE determinations are not feasible or give equivocal results with regard to the clinical history. Such an integrated strategy based on clinical history, skin tests, specific IgE levels and BAT responses will hopefully reduce the need for oral food challenges and guide clinicians in the appropriate selection of patients for dietary eviction of allergens.

Acknowledgements

We are grateful to all the children and their parents who participated in this study. We thank F. Vermeulen for her contribution in the recruitment of patients, Y. Peignois and the technicians of the allergology and cytometry units from the immunobiology laboratory for their technical assistance. We thank Phadia for providing Ara h 2 ImmunoCap.

Financial support was given by the 'Région Bruxelles Capitale', 'Nutripôle project'.

References

- 1 Bindslev-Jensen C, Ballmer-Weber BK, Bengtsson U *et al.* Standardization of food challenges in patients with immediate reactions to foods – position paper from the European Academy of Allergology and Clinical Immunology. *Allergy* 2004; **59**:690–7.
- 2 Boyano MT, Garcia-Ara C, Diaz-Pena JM, Munoz FM, Garcia SG, Esteban MM. Validity of specific IgE antibodies in children with egg allergy. *Clin Exp Allergy* 2001; **31**:1464–9.
- 3 Sampson HA. Utility of food-specific IgE concentrations in predicting symptomatic food allergy. *J Allergy Clin Immunol* 2001; **107**:891–6.
- 4 Celik-Bilgili S, Mehl A, Verstege A *et al.* The predictive value of specific immunoglobulin E levels in serum for the outcome of oral food challenges. *Clin Exp Allergy* 2005; **35**:268–73.
- 5 Rance F, Abbal M, Lauwers-Cances V. Improved screening for peanut allergy by the combined use of skin prick tests and specific IgE assays. *J Allergy Clin Immunol* 2002; **109**:1027–33.
- 6 Hill DJ, Heine RG, Hosking CS. The diagnostic value of skin prick testing in children with food allergy. *Pediatr Allergy Immunol* 2004; **15**:435–41.
- 7 Verstege A, Mehl A, Rolinck-Werninghaus C *et al.* The predictive value of the skin prick test weal size for the outcome of oral food challenges. *Clin Exp Allergy* 2005; **35**:1220–6.
- 8 Sporik R, Hill DJ, Hosking CS. Specificity of allergen skin testing in predicting positive open food challenges to milk, egg and peanut in children. *Clin Exp Allergy* 2000; **30**:1540–6.
- 9 Eigenmann PA. Are specific immunoglobulin E titres reliable for prediction of food allergy? *Clin Exp Allergy* 2005; **35**:247–9.
- 10 Niggemann B, Rolinck-Werninghaus C, Mehl A, Binder C, Ziegert M, Beyer K. Controlled oral food challenges in children – when indicated, when superfluous? *Allergy* 2005; **60**:865–70.

- 11 Lidholm J, Ballmer-Weber BK, Mari A, Vieths S. Component-resolved diagnostics in food allergy. *Curr Opin Allergy Clin Immunol* 2006; **6**:234–40.
- 12 Mothes N, Valenta R, Spitzauer S. Allergy testing: the role of recombinant allergens. *Clin Chem Lab Med* 2006; **44**:125–32.
- 13 Ebo DG, Sainte-Laudy J, Bridts CH *et al*. Flow-assisted allergy diagnosis: current applications and future perspectives. *Allergy* 2006; **61**:1028–39.
- 14 De Weck AL, Sanz ML, Gamboa PM *et al*. Diagnostic tests based on human basophils: more potentials and perspectives than pitfalls. *Int Arch Allergy Immunol* 2008; **146**:177–89.
- 15 Shreffler WG. Evaluation of basophil activation in food allergy: present and future applications. *Curr Opin Allergy Clin Immunol* 2006; **6**:226–33.
- 16 De Weck AL, Sanz ML. For allergy diagnostic flow cytometry, detection of CD203c instead of CD63 is not at all an improvement in other hands. *Clin Exp Allergy* 2003; **33**:849–52.
- 17 Moneret-Vautrin DA, Sainte-Laudy J, Kanny G, Fremont S. Human basophil activation measured by CD63 expression and LTC4 release in IgE-mediated food allergy. *Ann Allergy Asthma Immunol* 1999; **82**:33–40.
- 18 Erdmann SM, Sachs B, Schmidt A *et al*. In vitro analysis of birch-pollen-associated food allergy by use of recombinant allergens in the basophil activation test. *Int Arch Allergy Immunol* 2005; **136**:230–8.
- 19 Ebo DG, Hagendorens MM, Bridts CH, Schuerwegh AJ, De Clerck LS, Stevens WJ. Flow cytometric analysis of in vitro activated basophils, specific IgE and skin tests in the diagnosis of pollen-associated food allergy. *Cytometry B Clin Cytom* 2005; **64**:28–33.
- 20 Erdmann SM, Heussen N, Moll-Sladowy S, Merk HF, Sachs B. CD63 expression on basophils as a tool for the diagnosis of pollen-associated food allergy: sensitivity and specificity. *Clin Exp Allergy* 2003; **33**:607–14.
- 21 Prahl P, Krasilnikof F, Stahl SP, Norn S. Basophil histamine release in children with adverse reactions to cow milk. Comparison with RAST and skin prick test. *Allergy* 1988; **43**:442–8.
- 22 Heycock E, Heatley RV, Shires SE, Littlewood JM. An in vitro test for cows' milk protein intolerance? *Scand J Gastroenterol* 1986; **21**:1245–9.
- 23 Nolte H, Schiotz PO, Kruse A, Stahl SP. Comparison of intestinal mast cell and basophil histamine release in children with food allergic reactions. *Allergy* 1989; **44**:554–65.
- 24 Ocmant A, Peignois Y, Mulier S, Hanssens L, Michils A, Schandene L. Flow cytometry for basophil activation markers: the measurement of CD203c up-regulation is as reliable as CD63 expression in the diagnosis of cat allergy. *J Immunol Methods* 2007; **320**:40–8.
- 25 Muraro A, Roberts G, Clark A *et al*. The management of anaphylaxis in childhood: position paper of the European academy of allergology and clinical immunology. *Allergy* 2007; **62**:857–71.
- 26 Sampson HA. Food allergy. Part 2: diagnosis and management. *J Allergy Clin Immunol* 1999; **103**:981–9.
- 27 Sicherer SH. Food allergy: when and how to perform oral food challenges. *Pediatr Allergy Immunol* 1999; **10**:226–34.
- 28 Flinterman AE, van Hoffen E, den Hartog Jager *et al*. Children with peanut allergy recognize predominantly Ara h2 and Ara h6, which remains stable over time. *Clin Exp Allergy* 2007; **37**:1221–8.
- 29 Van der Veen, Van Ree R, Aalberse RC *et al*. Poor biologic activity of cross-reactive IgE directed to carbohydrate determinants of glycoproteins. *J Allergy Clin Immunol* 1997; **100**:327–34.
- 30 Wensing M, Akkerdaas JH, van Leeuwen WA *et al*. IgE to Bet v 1 and profilin: cross-reactivity patterns and clinical relevance. *J Allergy Clin Immunol* 2002; **110**:435–42.
- 31 Bock SA, Munoz-Furlong A, Sampson HA. Fatalities due to anaphylactic reactions to foods. *J Allergy Clin Immunol* 2001; **107**:191–3.
- 32 Eberlein-Konig B, Varga R, Mempel M, Darsow U, Behrendt H, Ring J. Comparison of basophil activation tests using CD63 or CD203c expression in patients with insect venom allergy. *Allergy* 2006; **61**:1084–5.
- 33 Bernstein IL, Vijay HM, Perelmutter L. Non-responder basophils in highly ragweed-sensitive subjects. *Int Arch Allergy Appl Immunol* 1977; **55**:215–6.
- 34 Kulig M, Bergmann R, Klettke U, Wahn V, Tacke U, Wahn U. Natural course of sensitization to food and inhalant allergens during the first 6 years of life. *J Allergy Clin Immunol* 1999; **103**:1173–9.
- 35 Shreffler WG, Beyer K, Chu TH, Burks AW, Sampson HA. Microarray immunoassay: association of clinical history, in vitro IgE function, and heterogeneity of allergenic peanut epitopes. *J Allergy Clin Immunol* 2004; **113**:776–82.
- 36 Beyer K, Ellman-Grunther L, Jarvinen KM, Wood RA, Hourihane J, Sampson HA. Measurement of peptide-specific IgE as an additional tool in identifying patients with clinical reactivity to peanuts. *J Allergy Clin Immunol* 2003; **112**:202–7.
- 37 Ebo DG, Lechkar B, Schuerwegh AJ, Bridts CH, De Clerck LS, Stevens WJ. Validation of a two-color flow cytometric assay detecting in vitro basophil activation for the diagnosis of IgE-mediated natural rubber latex allergy. *Allergy* 2002; **57**:706–12.
- 38 Kochuyt AM, Van Hoeyveld EM, Stevens EA. Prevalence and clinical relevance of specific immunoglobulin E to pollen caused by sting-induced specific immunoglobulin E to cross-reacting carbohydrate determinants in Hymenoptera venoms. *Clin Exp Allergy* 2005; **35**:441–7.
- 39 Foetisch K, Westphal S, Lauer I *et al*. Biological activity of IgE specific for cross-reactive carbohydrate determinants. *J Allergy Clin Immunol* 2003; **111**:889–96.
- 40 Ebo DG, Hagendorens MM, Schuerwegh AJ *et al*. Flow-assisted quantification of in vitro activated basophils in the diagnosis of wasp venom allergy and follow-up of wasp venom immunotherapy. *Cytometry B Clin Cytom* 2007; **72**:196–203.