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Comparison of two basophil activation markers CD63 and CD203c in the diagnosis of amoxicillin allergy

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Clinical and Experimental Allergy

Summary

Background To confirm allergy to β -lactam (BL), a basophil activation test in flow cytometry based on CD63 up-regulation was described. CD203c is a more recent basophil activation marker and up to day there is no consensus about which marker is the more sensitive one. CD203c has not yet been evaluated in the diagnosis of BL allergy.

Objective The aim of the study was to compare the reliability of CD203c to CD63 for the diagnosis of amoxicillin (AX) allergy, which is nowadays the most frequent BL allergy.

Methods Twenty-seven patients with an immediate positive skin test (ST) to AX, 20 had had anaphylaxis with AX and 7 had urticaria and/or angioedema, were compared with 14 controls with no allergy to BL and to six patients with delayed positive ST to AX.

Results In the anaphylaxis group, AX induced up-regulation of CD203c in the basophils of 12 patients out of 20 (60%) and of CD63 in four patients (20%) ($P < 0.02$). Two patients out of seven with urticaria or angioedema had a positive result with CD203c and CD63. In patients who had anaphylaxis, ampicillin (AMP) induced CD203c up-regulation in eight out of 12 (67%) patients tested, and CD63 up-regulation in 4 out of 12 (33%) (all patients who had anaphylaxis could not be tested with AMP). False-positive results were observed with CD203c as well as CD63, and for 10 patients indeed this was confirmed by a negative drug provocation test. The origin of conflicting results between CD63 and CD203c might be at least the targeting of basophils based on anti-IgE labelling. Among IgE⁺ gated cells, by means of CD33, a marker of monocytes, a contamination up to 50% by monocytes was detected. In contrast to CD63, CD203c is an activation marker specific of basophils with a basal low-level expression in resting basophils. Thus, IgE and CD203c double targeting of basophils avoids the contamination by monocytes.

Conclusion CD203c seems to be a more sensitive activation marker of basophils than CD63 for the diagnosis of amoxicillin allergy.

Keywords amoxicillin, basophils, CD203c, CD33, CD63, drug allergy, flow cytometry

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Introduction

β -Lactam (BL) antibiotics are known to cause severe hypersensitivity reactions. Penicillin allergy is reported to occur in up to 10% of patients. However, when patients with a history of penicillin allergy are evaluated, more than 90% of them are found to have negative test results and they can tolerate the antibiotic safely [1]. The discrepancy between claimed and real BL allergy results from multiple factors, such as skin eruptions due to an underlying illness, chronic urticaria, other drugs such as an NSAID taken at the same time, or a loss of sensitivity that occurs naturally and gradually year by year when BLs are avoided in allergic patients [2].

In patients suspected to be allergic to a BL antibiotic, reliable diagnostic tests are needed, as a false-negative diagnosis can put these patients at risk for severe allergic reactions, including anaphylaxis. Skin tests (STs) are considered to be the gold standard for the diagnosis of BL allergy. However, one group has reported having observed 49 patients with a history of an immediate allergic reaction to a BL, a negative ST and no specific IgE, who nevertheless had a positive drug provocation test (DPT) [3]. On the other hand, a false-positive ST might deprive patients of these most potent antibiotics. Additionally, systemic reactions like anaphylaxis or urticaria occur in <2% of ST with BLs [4]. Thus, the drawbacks of ST make *ex vivo* tests desirable to confirm allergy.

The pattern of allergic reactions to BL is changing, semi-synthetic penicillins and cephalosporins having replaced penicillin G [5]. Since the 1960s, the strategy of diagnostic tests has been developed using known metabolites of penicillin G, including the major antigenic determinant, the penicilloyl group [6, 7] and the minor antigenic determinants (MDMS) [8]. Regrettably, the metabolites of semi-synthetic penicillins and cephalosporins are not well known and neither available for skin testing. Therefore, STs are done with commercially available drugs in accordance with the guidelines of the European Network for Drug Allergy (ENDA) [9].

Cross-reactivities between different families of BL are known, but they are not as frequent as was previously supposed [10, 11]. In a series of patients with a positive ST to amoxicillin (AX), 43% also reacted to ampicillin (AMP), but only 9% reacted to benzylpenicilloyl-poly-L-lysine and 9% to MDM [12]. A minor degree of cross-reactivity, usually <10%, exists between penicillins and cephalosporins [13, 14], and it mainly occurs when the side chains are the same [11, 15–17].

Depending on the time interval between drug administration and the onset of symptoms, allergic reactions are classified as immediate (IgE mediated), the most severe form being anaphylaxis and late reactions with cutaneous and/or visceral involvement. The strategy of diagnostic testing depends on the type of reaction that has occurred: for immediate reactions, ST are read at 20 min; additionally, specific serum IgE antibody can be determined, allergen-induced basophil activation can be assayed by flow cytometry (FC) [12, 18], and antigen-induced release of sulphidoleukotrienes from leucocytes can be measured [19]. However, at present there is no consensus on the use of *in vitro* tests for the diagnosis of BL allergy.

A marker of basophil activation, CD63 (granule-associated tetraspan), which is assayed by FC, has been investigated and compared to ST, and it was reported to be both less sensitive and less specific in the diagnosis of BL allergy [12, 18]. A more recently described marker of basophil activation, CD203c (ecto-nucleotide pyrophosphatase/phosphodiesterase, a type II transmembrane ectoenzyme) [20, 21] has not yet been evaluated in BL allergy. The aim of the present study was to compare these two markers to see if a CD203c FC assay would be more predictive of AX allergy than the CD63 assay.

Material and methods

Patients

Forty-seven patients referred to the Allergy Centre of the Hôpital Tenon, Paris, between 2002 and 2005 for evaluation of a history of drug hypersensitivity were included in the following groups.

Amoxicillin allergic patients. This group included 27 patients with an immediate positive ST to AX, 13 were positive in the skin prick test (SPT) and 14 in the intradermal skin test (IST). Twenty of these patients had had an anaphylactic reaction occurring between 10 and 45 min after ingestion of AX. The remaining seven had only urticaria and/or angioedema, which began in <1 h after AX intake. The patients were skin tested with AX, AMP and cefuroxime (CFX) with a median of 1 year after the allergic reaction, and the basophil activation test was done within 3 months after the ST.

Controls. There were two types of controls: negative controls and positive delayed controls. Negative controls comprised 14 patients suspected of having had a myorelaxant or NSAID-induced anaphylactic reaction and/or urticaria or angioedema. These patients had negative ST to BLs and a negative DPT with AX. Positive delayed controls comprised six patients with a cutaneous drug eruption induced by AX and positive delayed skin test (DST) to AX, four with drug-induced erythema, one with a maculopapular rash and one with exfoliative dermatitis; the latter patient also had hairy cell leukaemia.

The study protocol was in accordance with the local ethical committee guidelines and all subjects gave their consent before being included. Tests were done only for a diagnostic purpose.

Skin tests

ST, done as previously described [10, 13], were performed at least 6 weeks after the hypersensitivity reactions. Commercially available AX for IV use Clamoxyl[®] was purchased from GlaxoSmithKline, AMP and CFX were purchased from Panpharma SA, France. Before use, the antibiotics were diluted in sterile saline solution containing 0.5% phenol (Stallergenes, Fresnes, France). For prick ST, BLs were diluted to 2, 20 and 200 mg/mL. For intradermal ST, BLs were diluted to 0.2, 2 and 20 mg/mL. Prick STs were done with increasing concentrations of the antibiotic until a positive reaction was observed; injections with increasing doses were separated by a 20-min interval. If the prick ST was negative, intradermal ST was done on the volar surface of the forearm, beginning with 0.05 mL of the highest dilution. Prick ST with histamine (10 mg/mL) and phenol-saline diluent solution were used as positive and the negative controls, respectively. The reactions were read after 20 min. The cut-off value for a positive prick ST was a weal diameter ≥ 5 mm and at least half the diameter of the histamine control, associated with a flare ≥ 10 mm, and a negative response to the control phenol-saline solution. The cut-off for the intradermal ST was a weal diameter ≥ 6 mm and at least 70% of the histamine control [22].

Drug provocation test

Double-blind, placebo-controlled orally given BL tests were carried out when the STs were negative. Each patient received successively at 2-h intervals, one of four randomized capsules, two containing lactose (placebo), the third containing 200 mg AX and the fourth 500 mg AX. For the patients who had a positive ST to AX, CFX was used for the DPT. All the DPTs were done by the same practitioner (HG).

β -Lactams for flow cytometry

BLs were diluted to final concentrations of 1, 0.1, 0.01 mg/mL in the dilution buffer (2 mM MgCl_2 and 1.2 mM CaCl_2 in phosphate-buffered saline (PBS)). Bovine serum albumin at a concentration of 2 g/L (Eurobio, Les Ulis, France, ref. 0126) was added to this buffer immediately before being used. The dilution buffer was used instead of a BL as the negative control in two tubes, and calcium ionophore 2.5 $\mu\text{g/mL}$ (Sigma) was used as the positive control.

Antibodies

R-phycoerythrin-conjugated anti-CD33 monoclonal antibody (MAb), fluorescein isothiocyanate (FITC)-conjugated anti-CD63 MAb, R-phycoerythrin-conjugated anti-CD203c MAb and tandem dye R-phycoerythrin-cyanin 5.1-conjugated anti-CD45 MAb were purchased from Beckman-Coulter-Immunotech, Marseille, France. Biotinylated goat anti-human IgE polyclonal antibody and R-phycoerythrin-conjugated streptavidin were purchased from Vector Laboratories, Burlingame, CA, USA. FITC-conjugated streptavidin (S3762) was purchased from Sigma-Aldrich, St Louis, MO, USA, and ECD (tandem dye phycoerythrin-Texas red)-conjugated streptavidin from Beckman-Coulter-Immunotech.

The *BASIC* (BASophilic Isolated from blood and analysed by flow Cytometry) assay was done as previously described [23, 24]. Basophil-enriched leucocytes were isolated from lithium-heparin anti-coagulated peripheral blood by centrifugation at 500 g for 20 min at 20 °C on a layer of ficoll ($d = 1.077$). Because a substantial proportion of basophils were denser than lymphocytes, not only the lymphocyte layer but cells suspended in ficoll between lymphocytes and red cells were also harvested. Aliquots of 100 μL of the leucocyte suspension containing 10^6 leucocytes were mixed with 100 μL of antigen or dilution buffer and then incubated for 30 min at 37 °C in a CO_2 (7%) incubator, after which the cell suspensions were fixed by the addition of 50 μL of 1% paraformaldehyde in PBS.

The basophils were then triple labelled by adding 10 μL FITC-conjugated anti-CD63 antibody, 10 μL tandem dye R-phycoerythrin-cyanin 5.1-conjugated anti-CD45 antibody, and 1/50 diluted 50 μL biotinylated anti-IgE antibody. Basophils in another tube were triple-labelled using

the same antibodies, except that the anti-CD63 was replaced by 20 μL R-phycoerythrin-conjugated anti-CD203c. After mixing and incubating for 30 min at 4 °C, the cell suspensions were washed and pelleted by centrifugation at 500 g for 10 min at 20 °C. After this, biotinylated anti-IgE antibody in anti-CD63-containing tubes was labelled by adding 50 μL R-phycoerythrin-conjugated streptavidin diluted 1/100 and that in anti-CD203c-containing tubes was labelled by adding 50 μL FITC-conjugated streptavidin. Cells were incubated again for 20 min at 4 °C and then washed.

When the study began, quadruple labelling was not possible because not enough sorts of fluorochromes-conjugated antibodies were available, which meant that CD63 and CD203c labelling had to be done in different tubes. A similar protocol was recently published [25].

FC analysis of basophil surface markers was performed on an Epics XL flow cytometer (Beckman-Coulter). On the histogram defined by forward scatter and side scatter, the initial gating of cells was done by a bit map around lymphocytes and monocytes. The second gating was done around the brightest cells in the histogram where IgE- and CD45-labelled cells were represented. These cells previously had been identified as basophils [23]. In each assay, up-regulation of activation markers was measured on at least 200 basophils.

The tube-to-tube reproducibility

The tube-to-tube reproducibility was determined by labelling basophils of nine patients, in 12 tubes for each patient and at least 200 basophils were counted per tube. In our technical conditions, in non-stimulated control tubes, the percentage of basophils up-regulating CD203c or CD63 had a standard deviation (SD) of 3%. An increase in the number of basophils counted over 200 did not improve significantly the standard deviation. A cut-off value for positive results was chosen at 2 SD (6%) exceeding the value of the non-stimulated control tube.

Statistical analysis

Results of FC were analysed in blind, the operator not being informed of the patients' diagnosis. Conversely, the diagnosis of allergy was done before the results of FC were available. Statistical analysis was done with χ^2 and a two-tailed Fisher's test.

Results

We express FC results in percentage of basophils up-regulating an activation marker. The percentage of allergen-activated basophils was defined as the percentage of basophils up-regulating a marker minus the value of non-stimulated control tube (Fig. 1). For CD203c and CD63, in

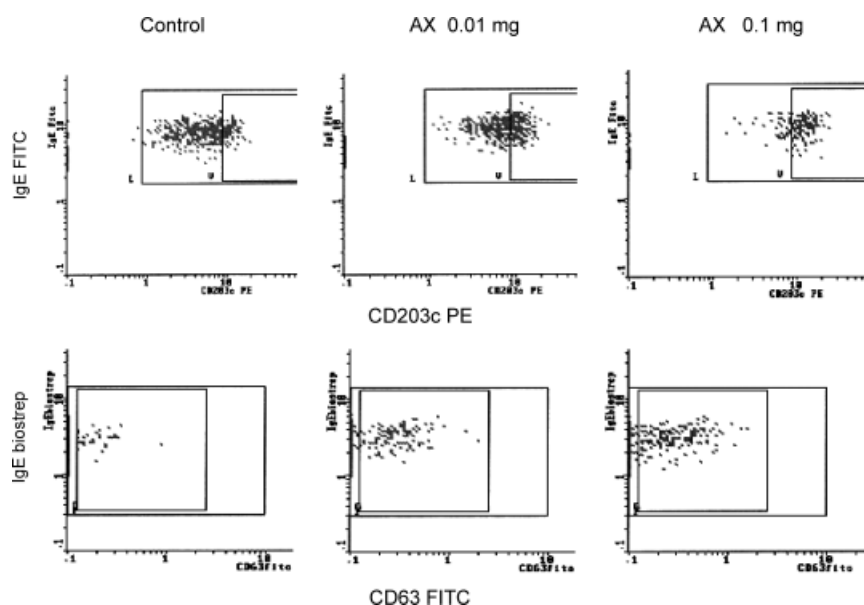


Fig. 1. Activation of basophils by amoxicillin (AX) in a patient who had previously anaphylaxis to AX. After incubation with AX, basophils are fixed and then labelled with anti-CD203c on the upper dot plots and anti-CD63 on the lower dot plots. Basophils were double-gated first by a bitmap on FS/SS dot plot, followed by a second bitmap on IgE/CD45 dot plot. In this patient, 50% of IgE-labelled cells were CD203c⁺ (the remaining CD203c⁻ cells are flattened on the ordinate axis). Incubation with 0.01 mg or 0.1 mg/mL of AX induced up-regulation of activation markers comparatively to the non-stimulated controls (10% of the basophils spontaneously up-regulated activation markers).

Table 1. Comparison of basophil activation markers CD63 and CD203c in the diagnosis of amoxicillin (AX) allergy

Groups of patients	Age (range)	Sex ratio	Flow cytometry		
			Ratio of patients with positive AX challenge		
			CD203c ⁺ *	CD63 ⁺	CD203c ⁺ or CD63 ⁺
Immediate ST ⁺					
AX anaphylaxis (<i>n</i> = 20)	44 (29–62)	10F/10M	12/20 (60%)	4/20 (20%)	14/20 (70%)
AX urtic/angio (<i>n</i> = 7)	50 (21–72)	4F/3M	2/7 (29%)	2/7 (29%)	3/7 (43%)
Total (<i>n</i> = 27)	46 (21–72)	14F/13M	14/27 (52%)	6/27 (22%)	17/27 (63%)
Negative controls (<i>n</i> = 14)	45 (25–62)	4F/10M	0/14 (0%)	3/14 (21%)	3/14 (21%)
DST ⁺ controls (<i>n</i> = 6)	50 (38–64)	5F/1M	0/6 (0%)	2/6 (33%)	2/6 (33%)

Immediate ST⁺: group of patients with immediate positive skin test to AX; AX anaphylaxis: patients recruited after AX induced anaphylaxis; AX urtic/angio: recruited after urticaria and or angioedema induced by AX.

Negative controls: controls with negative ST to AX, ampicillin, cefuroxime and negative drug provocation test to AX.

DST⁺ controls: group of patients with delayed positive ST to AX recruited after AX associated delayed cutaneous reactions.

**P* < 0.02 for CD203c vs. CD63 up-regulation in AX anaphylaxis patients' basophils incubated with AX. *P* < 0.001 for CD203c up-regulation in AX anaphylaxis patients' basophils incubated with AX vs. negative control patients' basophils incubated with AX.

non-stimulated control tubes, the SD of tube-to-tube variability was 3% (see 'Material and methods'), so the cut-off for positive results was defined to be 2 SD (6%) above the control tube. A test was reported positive when at least two dilutions of a BL gave a result equal or beyond the cut-off. Conversely, when a result equal or beyond the cut-off was observed at only one dilution, the assay was restarted with a new blood specimen.

Basophils of 27 AX allergic patients (all having a positive ST to AX, 20 with a history of anaphylaxis and 7 with a history of urticaria or angioedema) were incubated

with AX and analysed by FC. Twelve patients out of 20 (60%) with history of anaphylaxis had membrane up-regulation of CD203c on basophils, in contrast up-regulation of CD63 was observed in only four patients (20%) (Tables 1 and 2). The sensitivity of CD203c as activation marker was higher than CD63 (*P* < 0.02). Two patients out of seven with urticaria or angioedema had a positive FC with CD203c or CD63.

In negative control group (*n* = 14), any patient had a positive FC to AX when the marker was CD203c, and three had a positive FC when the marker was CD63. However,

Table 2. Comparison of flow cytometry results observed with amoxicillin (AX), ampicillin (AMP) or cefuroxime (CFX) between patients who had previously AX anaphylaxis vs. negative controls

Groups of patients/ allergen	Flow cytometry					
	CD203c			CD63		
	Ratio of positive*	% of activated basophils [†]		Ratio of positive	% of activated basophils	
		Mean	Range		Mean	Range
AX anaphylaxis						
AX (<i>n</i> = 20)	12/20	11	0–31	4/20	4	0–24
AMP [†] (<i>n</i> = 12)	8/12	8	0–37	4/12	6	0–25
CFX (<i>n</i> = 20)	3/20	3	0–17	2/20	2	0–21
Negative controls						
AX	0/14	0.1	0–2	3/14	2	0–7
AMP	0/7	0.9	0–3	2/8	4	0–14
CFX	0/8	0.2	0–2	2/8	2	0–8

*The cut-off for positive results (expressed as percentage of activated basophils) was defined to be 2 SD (6%) above the non-stimulated control tube.

†The percentage of activated basophils is the percentage of basophils expressing an activation marker minus the value of the non-stimulated control tube.

Some patients could not be tested with three BLs, because blood sample was not enough.

values were lower than those observed in anaphylaxis, and if the cut-off was chosen to be 3 SD instead of 2, they will be all negative. Moreover, the three patients had a negative DPT with AX (Table 3).

In the positive delayed reaction control group (n = 6), all patients had previously a drug eruption and were tested positive in delayed ST to AX. The percentage of positive FC results induced by AX in this group was similar to that of the negative control group (Table 1).

AX allergic patients were also tested with AMP and CFX. With AMP, 20 out of 27 (74%) patients were ST-positive. In patients who had anaphylaxis, AMP induced CD203c up-regulation in eight out of 12 (67%) patients tested, and CD63 up-regulation in four out of 12 (33%) (all patients who had anaphylaxis could not be tested with AMP). No patient with urticaria (n = 5) was found positive in FC with AMP. In contrast, with CFX, STs were negative (0/27); however, up-regulation was observed in three patients for CD203c and in two patients for CD63 out of 22 tested. These patients were submitted to a DPT with CFX, allergy was not confirmed (Table 3).

Conflicting FC results between CD203c and CD63 might be at least due to differences in targeting basophils. CD203c is not only an activation marker but it is also a specific marker of basophils, and when associated to anti-IgE, it improves targeting of basophils. The fraction of CD203c⁺ among IgE⁺ cells varies from 50% to 100%, depending on

Table 3. Drug provocation test (DPT) in patients with conflicting results: a positive flow cytometry result in a patient with negative ST to a BL

		% of activated basophils*				
Group of patients	Age Sex	CD203c		CD63		DPT†
		AX	CFX	AX	CFX	
Immediate ST AX ⁺ /CFX ⁻						
AX anaphylaxis	56 M	31	17	24	4	CFX ⁻
AX anaphylaxis	39 F	7	15	0	0	CFX ⁻
AX anaphylaxis	53 F	8	6	0	4	CFX ⁻
AX anaphylaxis	46 F	3	0	10	21	CFX ⁻
AX anaphylaxis	38 M	0	0	0	12	CFX ⁻
AX urticaria	51 F	29	0	1	8	CFX ⁻
Delayed ST AX ⁺ /CFX ⁻						
AX cutaneous reaction	38 F	0	0	0	10	CFX ⁻
Negative controls						
	45 M	0	2	7	0	AX ⁻
	49 M	1	0	6	0	AX ⁻
	40 F	0	0	8	8	AX ⁻

*Values above the cut-off value are in bold characters.

†Negative DPT with CFX or AX.

‡ST AX⁺/CFX⁻: skin test positive to AX but negative to CFX.

ST, skin test; AX, amoxicillin; CFX, cefuroxime.

the patient. As the meaning of this might be that IgE⁺ gated cells are not only basophils therefore, we tried to identify the contaminating cells by means of CD33, a marker of monocytes (Fig. 2). Two populations of CD33⁺ cells exist, monocytes, which are CD33 bright cells, and the basophils, CD33 dim cells (see 'Discussion'). In some patients, almost all IgE⁺ cells are CD33 dim; however, in others there is also a second population, IgE⁺ CD33 bright cells.

Discussion

In the diagnosis of AX allergy, our results with CD203c- or CD63-labelled basophils are divergent. In samples from patients with history of anaphylaxis, CD203c seems to be more sensitive than CD63 for the detection of AX-activated basophils. In patients with AX-induced urticaria, FC results were not different from controls, but this group was too small to be statistically significant. Immunologic response in anaphylaxis or urticaria seems different, ST positivity to benzyl penicillin was associated to urticaria, and positivity to AX, AMP and minor determinants with anaphylaxis [26]. However, the sensitivity of FC results with CD63 as activation marker or specific IgE determination to BLs was similar in anaphylaxis and urticaria [12, 18].

In an open preliminary study (unpublished) on 30 patients, we observed that CD63 was not enough sensitive for the diagnosis of BL allergy and we confirm this in the present study. Although our results were in agreement with published results [12, 18], the sensitivity of FC might be improved if patients were tested sooner after allergic

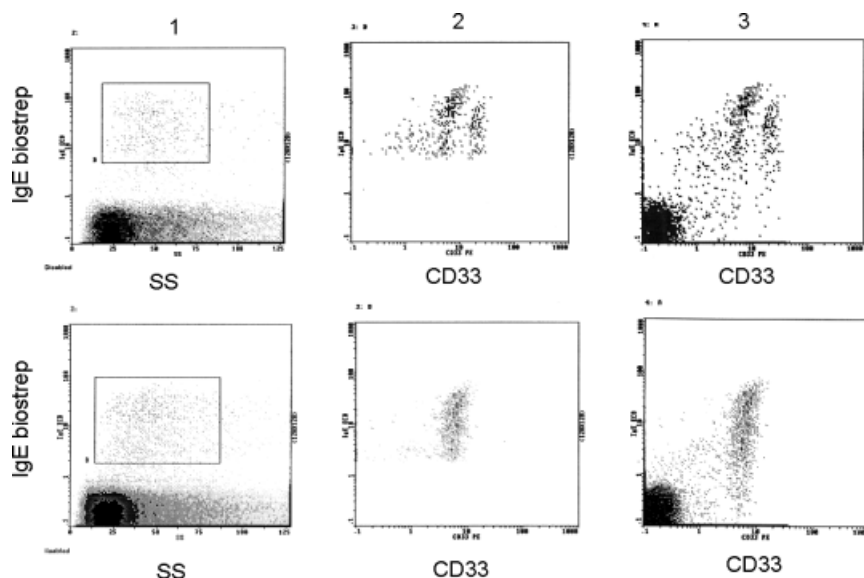


Fig. 2. IgE-targeted cells include some monocytes. Dot plot 2 (IgE/CD33) is gated on IgE⁺ cells in dot plot 1 [IgE/side scatter (SS)], dot plot 3 is not gated. On upper dot plots 2 and 3 (IgE/CD33), two columns of IgE⁺ CD33⁺ cells are observed. CD33 bright cells (right column) are monocytes (only the small fraction of monocytes labelled by anti-IgE is seen, the remaining fraction is flattened on abscissa axis). The IgE⁺ CD33 dim cells (left column) are basophils. On the lower dot plots, there is only one column, and these cells are IgE⁺ CD33 dim (basophils). Upper dot plots were observed in a patient having 60% of CD203c⁺ (a marker specific of basophils) among IgE⁺ cells, lower dot plots were observed in a patient with 95% of CD203c⁺ among IgE⁺ cells.

reaction. We tested the patients with a median of 1 year after the allergic reaction, and it was shown that a loss of ST sensitivity to AX occurs gradually year by year [2]. However, all the allergic patients that we selected were ST-positive, thus patients with a loss of ST sensitivity were not studied. It is not well known if the kinetics of loss of sensitivity to AX is different between ST and basophil up-regulation of activation markers.

The results of our previous study on muscle relaxant allergy contrasted with AX, where we found CD63 up-regulation to be both sensitive and specific enough for a diagnostic purpose [24]. Our results with CD63 in muscle relaxant allergy were confirmed, but on the other hand using CD203c, the sensitivity of FC was half that of CD63 [27]. This suggests that activation pathways of basophils might depend of the drug. In non-allergic patients, muscle relaxant drugs insufficiently diluted are activators of skin mastocytes [28] as well as of basophils with CD63 up-regulation [24], suggesting that a second activation pathway exists for these drugs. In fact, basophil or mastocyte can be activated by different pathways, mediated by FcεRI or other receptors [29].

Up to day, there is no consensus about which activation marker is more sensitive. CD63 is currently the most commonly used basophil activation marker. CD203c, more recently discovered, is used less often and in the diagnosis of drug-induced allergy there is only one report, relative to muscle relaxant allergy [27]. CD203c has been compared to CD63 in four studies. The first concluded that CD203c was a better marker than CD63 in the diagnosis of

latex allergy [21]; the second found CD63 more specific and CD203c more sensitive for bee or wasp allergy [30]; the third concluded that CD63 was more sensitive than CD203c in the diagnosis of muscle relaxant drug allergy [27] and the fourth found CD203c as reliable as CD63 in the diagnosis of cat allergy [25].

Discrepancies about the sensitivity of CD203c compared to those of CD63 might have different explanations. Contradictory conclusions were obtained using allergens that were more or less potent activators of basophils. Suboptimal activation of basophils might produce dissociation of up-regulation of markers. On the other hand, different technical conditions in handling cells or targeting basophils might also be an explanation.

In fact, CD63 and CD203c activation pathways and time kinetics are very different. CD203c molecules may be located in vesicles near the membrane, whereas CD63 is stored in granules [30]. Inhibitors of hydroxymethylglutaryl coenzyme A reductase, or phosphoinositide-3-kinase, inhibit IgE-mediated histamine release and CD203c up-regulation but not that of CD63 in basophils. A stimulator of the protein kinase C pathway induce CD203c up-regulation rapidly, within 1 min of stimulation, but CD63 up-regulation is slower, reaching a maximum only after 60 min [30, 31]. Incubation time with allergens and especially with BL varies from 10 to 40 min depending on the study [12, 18]. In our assay, the incubation time with allergens before labelling with CD63 or CD203c was 30 min. The duration of incubation might not be optimal for the expression of each of these markers,

perhaps for CD203c a shorter time would be more adequate; nevertheless, identical incubation time avoided introduction of differences in the results due to changing technical conditions.

Specificity against basophils of the activation marker might also explain discrepancies. CD63 is also an activation marker for platelets and leucocytes, and adhesion of activated platelets to basophils might disturb CD63 analysis of basophils [29]. On the other hand, CD203c is not only an activation marker but also a specific marker of resting basophils [30], and in association with anti-IgE, it improves targeting of basophils [21]. In some studies, basophils are targeted only with anti-IgE. However, all IgE⁺ gated cells are not CD203c⁺, which might indicate a contamination by cells other than basophils. In order to verify that the remaining cells were monocytes, we labelled them with anti-CD33, a marker of monocytes. In fact, among CD33⁺ cells, depending on fluorescence intensity, two populations might be identified: CD33 bright cells, which are monocytes, and CD33 dim cells, which include the subpopulation of CD16⁺ monocytes [32] and the basophils [33]. Among CD33 bright cells, there is the subpopulation of CD2⁺ monocytes [32], precursors of dendritic cells, which are IgE⁺ because they express FcεRI [34]. Our results confirm and extend this; IgE⁺ gated cells include CD33 dim cells, which are basophils, and CD33 bright cells, which are monocytes. The proportion of monocytes in IgE gated cells varies largely, from 0% to 50%, depending on the patient. This variability might correlate with the serum IgE level, which regulates the expression of FcεRI and the binding of IgE on the monocytes membrane [35]. Contamination by monocytes of IgE gated cells underscores the percentage of activated basophils on one hand, and on the other hand because activated monocytes also express CD63, false positive results might be generated. Therefore, a specific targeting of basophils is essential.

In contrast to CD203c, we did not find a significant correlation between ST results with AX and positive CD63 results by FC. Our results with CD63 do not contradict others, in patients with a positive history of BLs allergy near half of the patients were found positive by FC using CD63 as marker, independently the ST was positive or negative [12]. A similar result was reported in a European multicentric study on BL allergy including 181 patients, presented in the European Network for Drug Allergy (ENDA) Congress (Vienna, June 2006) (not yet published, the report is available at Buhlmann Laboratories, Switzerland). A positive FC in ST-negative patient was explained by the occurrence of a false-negative ST. However, the results of DPT, negative in our patients with a conflicting positive FC result to a negative ST, do not seem to confirm this hypothesis. Moreover, our results with DPT underline that false-positive results exist with CD63 as well as with CD203c. Up until recently, false-positive results in FC

were unknown, they were described in the diagnosis of latex allergy [36].

In contrast to the availability of data on the frequency of false-negative ST in BL-allergic patients, we do not know the real frequency of false positive ST results in such patients, because for ethical reasons, oral provocation tests are not done in patients with a definitely positive ST. Although the ST remains the gold standard, false-positive BL-ST certainly do occur and comparison to ST of the basophil activation test underscores its sensitivity as a means of excluding BL allergy in patients who require one of these drugs. False-positive and negative results might explain the moderately good correlation between tests; however, using CD203c instead of CD63 improves the concordance of FC with ST.

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