

Marked improvement of the basophil activation test by detecting CD203c instead of CD63

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Summary

Background The flow cytometric basophil activation test by detection of CD63 expression has been developed as an alternative method for *in vitro* diagnosis of IgE-mediated reactions to various allergens. Despite promising initial studies, the test remains disappointing in terms of sensitivity. CD203c has recently been demonstrated as a specific activation marker of basophils that is rapidly up-regulated after allergen challenge in sensitized patients.

Objective The goal of the present study was to compare basophil activation tests by using either CD203c or CD63 in the diagnosis of immediate-type allergy to latex.

Methods Twenty-seven patients (health care workers of our institution) who developed clinical features evocative of allergy after contact with latex were included and classified into two groups. Group 1 ($n = 16$) comprised true allergic patients who presented with typical signs of immediate allergic reaction associated with a positive skin test (prick test). Group 2 ($n = 11$) consisted of patients whose clinical history was not typical and had negative skin test. Twelve healthy subjects were also studied as controls. We compared the sensitivity of two triple-staining flow cytometric protocols measuring basophil activation after latex stimulation: CD45-IgE-CD63 and CD45-IgE-CD203c.

Results The CD203c protocol showed a higher sensitivity than the CD63 protocol (75% vs. 50%). In comparison, latex-specific IgE sensitivity was found to be 69%. Furthermore, the magnitude of the basophil response was significantly higher with CD203c in comparison with CD63. Specificity was 100% for both protocols.

Conclusion Due to superior gating of basophils and a higher range of activation in response to allergen, the basophil activation test is markedly improved by use of CD203c instead of CD63.

Keywords basophils, CD63, CD203c, flow cytometry, latex allergy

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Introduction

For several years, the incidence of allergy and anaphylaxis has been increasing in Western Europe, North America and Australia [1]. Anaphylaxis consists of an immediate IgE-dependent reaction in response to allergens. It is caused by an initial systemic histamine release by mast cells and basophils that may lead to shock with laryngeal oedema, lower-airway obstruction and hypotension. The most frequent allergens involved in immediate allergy are found in peanuts, fish, bee and wasp venoms, drugs and latex [2]. The diagnosis is mainly based upon an evocative clinical history, positive skin tests, which remain the gold standard in this context and, if available, detection of specific IgE [3]. In most patients, these features allow both diagnosis and identification of the responsible allergen. In the remaining cases, functional *in vitro* tests are necessary.

These are focused on circulating basophils, as these cells are considered to be equivalent to tissue mast cells. Moreover, basophils themselves play a key role in immediate allergic reactions [4–8]. The first approach to basophil functional studies was the histamine release test. However, its clinical benefit has remained controversial due to insufficient sensitivity and specificity [3, 9, 10]. Recently, our group and others took advantage of flow cytometry to develop new tools for monitoring basophil activation upon allergen challenge by detecting surface expression of CD63 [11–13]. This protocol has been validated for common allergens and has shown promising results [14–17]. Nevertheless, with respect to drug allergy, which constitutes the main indication for this kind of test, three independent studies reported similar low sensitivities ranging between 50 and 64%, which is not sufficient in terms of clinical usefulness [12, 18, 19]. Consequently, in a recent study on allergy to muscle relaxant drugs, we concluded that an activation marker that is more specific than CD63 would be desirable [19].

CD203c (ecto-nucleotide pyrophosphatase/phosphodiesterase 3), a type II transmembrane protein, has been described as being selectively expressed on basophils, mast cells and their

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CD34⁺ progenitors [20, 21]. As CD203c is rapidly up-regulated after allergen challenge in sensitized patients, it has been proposed as a new tool for allergy diagnosis [20, 22].

The objective of the present study was to compare basophil activation tests using either CD203c or CD63 in the diagnosis of immediate-type allergy. We have chosen to work on latex allergy because its diagnosis is readily established on the basis of clinical symptoms, skin testing and specific IgE [23]. Hence, it allows the definition of a well-characterized allergic patient group, which permits a reliable assessment of the sensitivity of basophil activation tests.

Subjects and methods

Subjects

Twenty-seven patients were referred to the Employee Health Department of our hospital (Lyon-Sud University Hospital, France) for investigation of clinical symptoms evocative of an allergy to latex. After giving informed consent, these subjects were divided into two groups. Group 1 consisted of 16 truly allergic patients who developed typical features of an anaphylactic reaction after contact with latex (usually latex gloves). Allergy diagnosis was confirmed by positive skin testing (prick-test). Group 2 consisted of 11 patients whose clinical signs were less evocative. Allergy was ruled out on the basis of negative skin testing. We also included 12 non-allergic healthy individuals from our laboratory. These subjects had regular exposure to latex without any clinical signs suggestive of sensitization and negative latex specific IgE.

Antibodies

The following monoclonal antibodies were used according to the manufacturers' recommendations: phycoerythrin (PE)-conjugated anti-human CD63 (20 µL/100 µL whole blood, clone CLBGran/12, IgG1 mouse; Immunotech, Marseille, France), PE-conjugated anti-human CD203c (20 µL/100 µL, clone 97A6, IgG1 mouse; Immunotech), phycoerythrin-cyanin 5 (PC5)-conjugated anti-human CD45 (10 µL/100 µL; Dako, Glostrup, Denmark). Surface bound IgE was detected using diluted polyclonal fluorescein isothiocyanate (FITC)-conjugated anti-human IgE (dilution one-half of a 0.5-mg/L solution; Caltag Laboratories, San Francisco, CA, USA).

Cell preparation

The test was carried out on heparinized blood within 3–4 h after sampling. For each sample, we prepared both negative controls [10 µL of Ca/Mg-free phosphate-buffered saline (PBS), Gibco BRL, Grand Island, NY, USA, added to 100 µL of blood without allergen] and positive controls (10 µL of formyl-methionyl-leucyl-phenylalanine (fMLP, Sigma, St Louis, MO, USA) to give a final concentration of 10⁻⁶ M after addition to 100 µL of whole blood). Aliquots (100 µL) of each blood sample were incubated with a series of dilutions (10 µL of each) made by addition of PBS to a latex stock solution prepared by mixing two products (1:1) commonly used for skin testing (Stallergènes SA, Anthony, France and Allerbio, Paris, France). The following dilutions were made: 1/100, 1/500, 1/1000, 1/5000. After mixing blood and allergen, controls and samples were incubated at 37 °C for 10 min. The reaction was stopped on

ice, followed by a 30-min staining performed at +4 °C in a dark chamber. Finally, samples were lysed using either the Q-prep system (Beckman-Coulter, Hialeah, FL, USA) for the CD63 protocol or the Lysing Whole Blood Reagent (Beckman-Coulter) for the CD203c protocol. The specificity of the CD203c protocol was assessed by incubation of the same dilutions of the latex solution with blood from non-allergic healthy individuals.

Flow cytometry

Samples were analysed on a Coulter EPICS XL flow cytometer (System II software, Beckman-Coulter) which was calibrated daily with Flow-Check fluorospheres (Beckman-Coulter). For CD63, we used the protocol previously established [19]. We initially defined a region with side-scatter characteristics and CD45 expression that comprised basophils along with some lymphocytes and monocytes (Fig. 1a). Then, among these cells, we isolated basophils on a biparametric histogram on the basis of expression of CD45 and surface bound-IgE. Basophils constituted a small distinct population of IgE positive cells (Fig. 1b) in which we examined CD63 expression before and after latex challenge. We also set up a protocol for investigating CD203c expression using tricolour staining with anti-CD45-PC5, anti-IgE-FITC and anti-CD203c-PE. As described above, we initially gated cells using a CD45-SSC biparametric histogram (Fig. 1c). Then, we readily defined basophils on a CD203c-IgE biparametric histogram on the basis of expression of both markers (Fig. 1d). We then examined CD203c expression in the basophil population before and after latex challenge (Fig. 2). For both protocols, we expressed the results as the percentages of basophils that expressed CD63 or CD203c above a threshold level as defined by the quadrant in Fig. 2. The thresholds were defined on the basis of the negative control without any latex. Alternatively, the results were expressed as the mean of fluorescence intensities (MFI) related to the entire basophil populations. Flow cytometric results were considered positive when at least two sequential dilutions induced more than 10% CD63- or CD203c-positive basophils above control values as previously established [11, 19].

Measurement of total and specific serum IgE

The concentrations of total and latex-specific IgE [radioallergosorbent test (RAST)] were determined by fluorescence enzyme immunoassay (UniCAP, Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions. Values of allergen-specific IgE above 0.35 kU/L were considered as positive.

Statistical analyses of the data

Data were expressed as mean ± SEM. The statistical significances between protocols were determined by the non-parametric Mann-Whitney test.

Results

Twenty-seven subjects were included in the study. Patients' details (sex and age) and all biological data are listed in Table 1. Among these patients, we documented 16 to be allergic to latex (group 1), whereas 11 were classified as non-allergic (group 2). Total IgE concentrations were not significantly

Fig. 1. Basophil identification by two flow cytometric protocols using either CD63 or CD203c as activation marker. CD63 protocol: (a) Leucocyte biparametric representation on the basis of side-scatter and CD45 staining. Gating region (α) is defined from the usual basophil localization which lies between monocytes and lymphocytes. (b) Gated cells from α expressed on the basis of PC5-CD45/FITC-IgE staining. Basophils constitute a population (IgE bright) above lymphocytes and monocytes (one representative patient). CD203c protocol: (c) Leucocyte biparametric representation (SSC-CD45) excluding debris (on the left of the dot-plot). As in (a), gating region (β) is defined around the usual basophil localization. (d) Gated cells from β expressed on the basis of PE-CD203c/FITC-IgE. Basophils (IgE++ and CD203c+) are readily distinguished from other cells as they co-express IgE and CD203c (one representative patient).

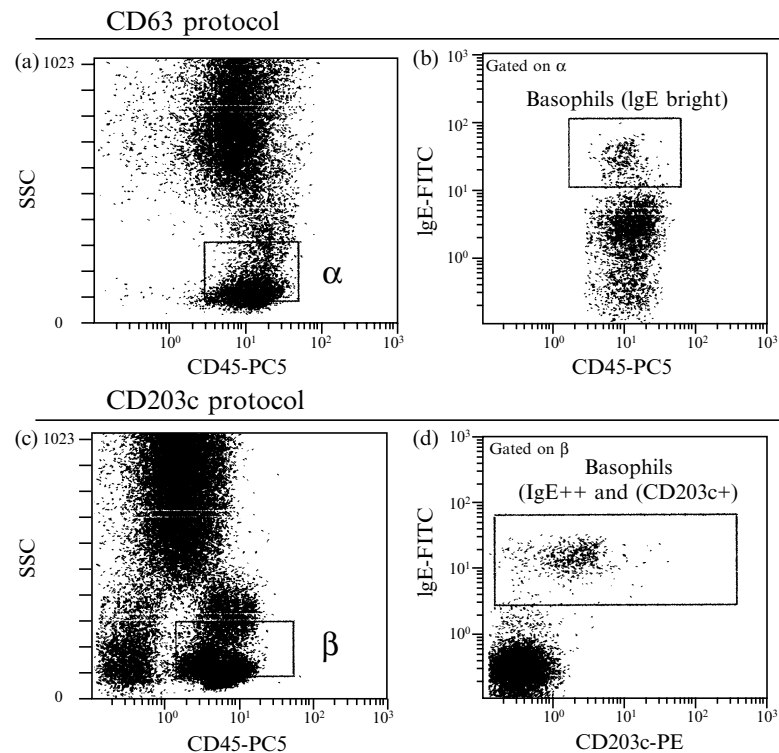
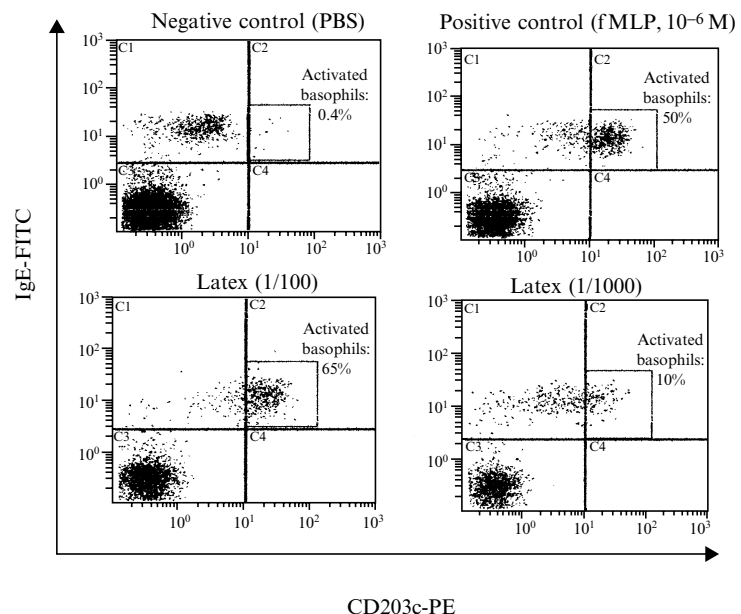


Fig. 2. Representative increased expression of CD203c after fMLP or allergen challenge. Gated cells from Fig. 1(c) are presented on the basis of CD203c-IgE staining: before stimulation (upper left dot-plot), after FMLP challenge (upper right) and after latex challenge at two different concentrations (lower dot-plots). Results from one representative patient of 12.



different between the two groups. In group 1, 11 subjects out of 16 presented with positive specific-IgE levels, ranging from 0.4 to 15.2 kU/L. In the five remaining patients, RAST were below the threshold of 0.35 kU/L. Hence, the sensitivity of latex-specific IgE was 69% which is very similar to results previously reported for the diagnosis of immediate allergy to latex [24, 25].

Concerning cell gating by flow cytometry, we initially observed that it was not possible correctly to define basophils on the sole basis of their basal CD203c expression as initially proposed by Platz et al. [22]. In about 40% of our samples (from both patients and donors), an appreciable number of resting basophils only weakly expressed CD203c, and on this basis could not be distinguished from other cells. These cells

Table 1. Patients data, total IgE, latex-specific IgE, CD63 and CD203c results in 27 patients with clinical signs suggestive of allergic reactions to latex

	Patient	Sex	Age (year)	Total IgE (kU/L)	Specific IgE (kU/L)	CD63 (% activation)	CD203c (% activation)
Allergic	1	F	44	6	< 0.35	13	66
	2	F	30	17	< 0.35	12	18
	3	M	38	nd	10.3	12	1
	4	F	29	99	1.57	43	82
	5	F	52	134	< 0.35	7	5
	6	F	47	18	0.4	13	22
	7	F	42	80	1.6	0	19
	8	F	39	65	< 0.35	12	54
	9	F	41	942	1.6	25	70
	10	F	50	195	1.45	45	91
	11	F	35	196	13.6	4	43
	12	F	39	3689	1.55	11	44
	13	F	50	34	0.48	35	24
	14	F	33	383	< 0.35	0	0
	15	F	37	5289	15.2	1	2
	16	M	19	12	1.6	29	48
Non-allergic	17	F	47	9	< 0.35	0	0
	18	F	48	14	< 0.35	0	0
	19	F	45	196	< 0.35	1	1
	20	F	47	1968	< 0.35	0	0
	21	F	33	31	< 0.35	0	0
	22	F	56	274	< 0.35	1	3
	23	M	45	19	< 0.35	5	1
	24	F	45	159	< 0.35	0	6
	25	F	42	128	< 0.35	0	1
	26	F	37	65	< 0.35	0	1
	27	F	56	53	< 0.35	1	1

Flow cytometric results are expressed as the highest activation value obtained among four dilutions of latex. nd, not done.

would have been excluded if we had defined the basophil population only on the basis of anti-CD203c and side-scatter (Figs 1d and 2). In contrast, the co-expression of both CD203c and IgE permitted identification of basophils with a high recovery. Thus, we used this latter gating strategy for the CD203c protocol.

Basophils from all of the subjects tested responded positively to fMLP on the bases of expression of both CD63 and CD203c, demonstrating the capacity of all of the cell preparations under study to respond to stimulation (Table 2). However, the extent of the response to fMLP with the CD203c protocol was over twice that obtained with the CD63 protocol ($P < 0.02$). There were no significant differences between the two groups of subjects in their responses to fMLP (Table 2).

Basophils from most of the allergic patients underwent a marked activation in response to a latex challenge (Fig. 2). This effect was concentration-dependent, suggesting a specific biological mechanism (Fig. 3). We considered the activation test to be positive when at least two sequential latex dilutions induced an increase of more than 10% above control levels in CD63- or CD203c-positive basophils. By this criterion, only eight of the 16 allergic patients were identified as being allergic on the basis of the CD63 protocol. In sharp contrast, we have been able to classify 12 of these patients as allergic when using the CD203c protocol (Fig. 4). Consequently, the sensitivities of the CD63 and CD203c protocols were 50 and 75%, respectively. Regarding specificity, none of the non-allergic patients

Table 2. Basophil activation in response to fMLP (10–6 M) as measured in two distinct basophil activation tests using either CD203c or CD63 as activation marker

Percent activated basophils (CD63 ⁺ or CD203c ⁺) above control values		
	Allergic patients (n = 16)	Non-allergic patients (n = 11)
CD203c	41 ± 7* (range 12–74)	50 ± 6* (range 14–85)
CD63	18 ± 4 (range 5–43)	23 ± 6 (range 2–53)

Results are expressed as mean ± SEM. * $P < 0.02$ vs. the CD63 protocol Mann–Whitney non-parametric test.

exhibited basophil activation with either protocol in response to a latex challenge. Likewise, we only found negative results in the 12 healthy subjects (data not shown) reflecting the absence of non-specific basophil activation. Thus, the specificity was of 100% in both protocols. As some authors have proposed thresholds for positivity at 5% [15, 18] and 15% [14, 16], we also investigated whether different threshold levels could improve sensitivity without decreasing specificity. However, at 5%, as illustrated in Fig. 4 for CD203c, a lower cut-off value would have given positive results among non-allergic patients, thus decreasing specificity. On the other hand, at 15%, the sensitivity of the CD203c protocol was not altered whereas that of the

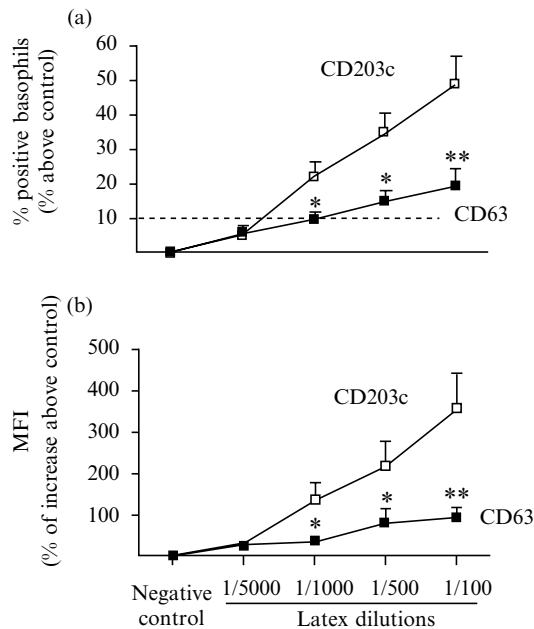


Fig. 3. Effects of latex dilutions on CD203c and CD63 expression by basophils from allergic patients. The values are presented as: (a) % of CD63- or CD203c-positive basophils above control values, or (b) the mean of fluorescence (MFI) related to the entire basophil population expressed as percentage above control values. Results are means $\pm$ SEM ($n = 12$). * $P < 0.05$, ** $P < 0.005$ (Mann-Whitney non-parametric test). The results were considered as positive when at least two sequential dilutions induced more than 10% CD63- or CD203c-positive basophils above control values [dashed line in (a)].

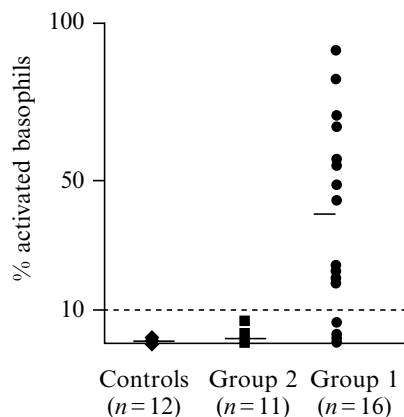


Fig. 4. Levels of activated basophils in individual subjects following latex challenge. Each point represents the percentage of activated basophils obtained in each patient or donor after challenge with the highest concentration of latex solution (1/100). Horizontal lines are medians. Dashed line (10%) represents the threshold for positive results. Group 2 comprised non-allergic patients; group 1 comprised allergic patients.

CD63 protocol was diminished (44%). We also analysed the results on the basis of the percentage increase in MFI instead of the percentage of positive basophils. Using a 40% increase to define a positive result (i.e. a 1.4-fold increase in MFI) we found that the sensitivity for the CD63 protocol was slightly reduced for CD63 (44%), whereas that for the CD203c protocol remained constant at 75%.

It is noteworthy that upon allergen challenge, we obtained significantly higher basophil responses with the CD203c

protocol on the basis of both the percentage of positive cells and the percentage increase in MFI (Fig. 3), thus further increasing the reliability of this approach.

Discussion

Flow cytometric protocols have been developed for investigating basophil activation upon allergen challenge. This approach relied on two main characteristics of basophils: the recognition through the expression of IgE on their surface and the monitoring of their activation by detecting CD63. However, these characteristics are not specific to basophils. This may explain why, when applied to drug allergy, these tests have remained disappointing in terms of sensitivities ranging between 50 and 64% [12, 18, 19]. The objective of the present study was to assess a new basophil activation test in the diagnosis of immediate-type allergy by use of CD203c rather than CD63 as an activation marker for basophils. This resulted in a marked improvement in the basophil activation test.

In order to define a group of truly allergic patients, we have chosen to work on latex allergy for several reasons. It constitutes a typical IgE-mediated hypersensitivity. This form of allergy has affected a large number of individuals in recent years and has become an important health concern, especially in health care workers at the hospital. Its diagnosis is in most cases readily established on the basis of evocative clinical symptoms (including urticaria, rhinitis conjunctivitis, asthma and anaphylaxis associated with exposure to latex), skin testing (prick) and latex-specific IgE if necessary [23, 26]. This allows the definition of a well-characterized allergic patient group, thus permitting a reliable comparison of the basophil activation tests.

We included 27 patients presenting with clinical signs suggestive of latex allergy. All were health care workers from our hospital. Among them, 16 were considered as true allergic patients because they developed typical features of an anaphylactic reaction after contact with latex (most commonly latex gloves). Allergy diagnosis was also established on the basis of positive prick tests. In terms of sex ratio, total circulating IgE levels and sensitivity of latex-specific IgE (69%), our results are quite similar to those published by others in recent studies [24, 25, 27]. The main point of the present report is to demonstrate an increase in the sensitivity of the basophil activation from 50% with CD63 to 75% with CD203c in this well-defined group of allergic patients. This study supports the recent findings of Hauswirth et al. [28] and constitutes the first description of such a comparison between these kinds of flow cytometric tests in terms of clinical practice. Furthermore, it is noteworthy that the test would probably be improved by the use of recombinant allergens [28].

The improved sensitivity in the basophil activation test using CD203c expression may be due to two factors. First, the recognition of basophils is better with the use of CD203c. Even if it is not possible to identify all resting basophils on the sole basis of their basal CD203c expression, as was initially proposed by Platz et al. [22], the double staining of both IgE and CD203c permits basophil identification with a high recovery. Indeed, the identification of basophils using prior protocols relied on a single IgE-labelling, although it is known that FcεRI expression can vary considerably on cell surfaces from one patient to

another [29]. This may explain why in some cases basophils were difficult to identify and sometimes totally missing. The difference in the CD203c levels of expression between the study reported by Platz et al. [22] and ours may be explained by differences in the two protocols. They stained basophils at room temperature for 15 min and lysed red cells using the Iotest lysis reagent (Immunotech), whereas we labelled cells at 4 °C for 30 min and used the Whole Blood Lysing Solution (Beckman-Coulter) as recommended by the manufacturer. Whether different procedures could lead to discrepant results for CD203c measurement remains to be investigated.

The second reason for the improved sensitivity with the CD203c is due to the greater increase in expression of CD203c compared to CD63. In sensitized patients, basophils increased their CD203c levels up to 350% above control values in response to allergens, whereas the increase in CD63 was below 100% (Fig. 3b). Similar results were obtained when expressing the results as CD203c- or CD63-positive basophils. Even with the highest concentration of latex (1/100), the mean percentage of CD63-positive basophils was below 20% while that of CD203c-positive basophils was 48% (Fig. 3a), allowing a clear distinction between resting and activated basophils. The increased sensitivity of the CD203c protocol is further supported by the data obtained upon fMLP challenge, in which case the CD203c protocol gave significantly enhanced results (Table 2). In summary, both superior gating and a higher range of activation in response to allergen contribute to a marked improvement in the basophil activation test when using CD203c rather than CD63.

CD203c corresponds to a surface antigen expressed on human basophils recently recognized by the monoclonal antibody 97A6 [20]. This antigen, belonging to the type II transmembrane protein family, is a multifunctional ecto-enzyme called ectonucleotide pyrophosphatase phosphodiesterase 3 (E-NPP3) [21] that catalyses the cleavage of a number of molecules, including deoxynucleotides and nucleotide sugars [30, 31]. In addition, E-NPP3 contains also a somatomedin B-like domain and a cell adhesive motif [32], but their potential functions remain totally unknown with respect to basophil physiology. CD203c appears to be selectively expressed on the basophil/mastocytes lineage [20]. To date, no other cells from human peripheral blood have been reported to express this marker [31]. Its expression on basophils is rapidly up-regulated after stimulation with appropriate allergen in sensitized patients to acarids or hymenoptera or after a cross-linking of FcεRI with anti-IgE antibodies [20, 22]. Importantly, no effect was observed on CD203c expression when basophils were challenged by a large number of cytokines, including Th2 mediators such as IL-4, IL-5, IL-9 or IL-13 [20]. This suggests that CD203c up-regulation is more or less specific to the cross-linking of FcεRI or to the effects of chemoattractants such as fMLP. The rapid rise in CD203c expression within several minutes of basophil stimulation indicates the constitutive presence of intracellular stores of this protein. Consequently, CD203c might play a pivotal role in IgE-dependent activation of basophils. However, there is as yet no evidence demonstrating an involvement of CD203c in allergic mechanisms. In particular, whether or not CD203c is involved in basophil degranulation (i.e. histamine release, CD63 up-regulation) remains to be elucidated.

In conclusion, we confirm that CD203c is a basophil activation marker showing a very large increase in expression in response to allergens. Although identification of the basophil

population solely on the basis of staining with anti-CD203c is not practical, measurement of this marker clearly improves the basophil activation test in comparison to the previously used protocol based on CD63. Sensitivity was significantly improved, whereas specificity remained at 100%. Both the improved identification of cells as well as the higher degree of activation in response to allergen may contribute to simplify the interpretation of results. Measurement of basophil expression of CD203c constitutes a critical step for performing standardized measurements of basophil activation. However, the definition of a common gating strategy as well as the setting of threshold value remains to be established for assessing this kind of test in multicentre studies. In particular, the clinical benefit of the measurement of CD203c expression has now to be investigated in drug allergy where there is still a need for functional *in vitro* techniques when skin testing cannot be performed, or specific IgE are not commercially available, or in patients for whom clinical and laboratory evidence remains discrepant.

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