

Analysis of anti-IgE and allergen induced human basophil activation by flow cytometry. Comparison with histamine release

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Abstract. *Objective and Design:* On the basis of flow cytometric methods previously described for the analysis of human basophil activation, we present here a bi-color anti-IgE FITC, anti CD63 PE method and the correlation with histamine release.

Materials and Subjects: Subjects allergic to grass pollen were selected by their clinical history, skin tests and specific IgE.

Methods: Basophils gated in the lymphocyte region of the side scatter (SSC)/forward scatter (FSC) pattern were selected by their high IgE epitope density. Percentage of cells expressing CD63 marker, upregulated on activated basophil membrane, was calculated by the cytometer. Histamine released into the supernatants was measured by RIA.

Results: In these conditions, flow cytometric analysis of blood leukocytes showed that the selected cells had the phenotype CD14[−], CD19[−], CD45⁺, IgE⁺⁺ and CD63[−] or ⁺ which is related to human basophil phenotype, the isotype controls being negative. The use of an anti-CD41 FITC antibody also showed the presence of aggregated platelets on the basophil membrane, CD63 antigen being, however, expressed by basophils themselves and not by platelets. Moreover, no statistical difference was observed between histamine release and flow cytometry after passive sensitization of blood donor leukocytes.

Conclusion: Flow cytometry, as a popular method often used in the immunology and haematology departments of clinical laboratories may represent a new alternative for allergy diagnosis and basophil pharmacology.

Key words: Flow cytometry – Histamine release – Human basophil activation – CD 63 – Allergy diagnosis

Basophil activation is induced by both IgE-dependent (e.g. anti-IgE antibody, specific allergen) and IgE-independent (e.g. neuropeptides, major basic protein from eosinophils) mechanisms. It is modulated by cytokines (e.g. IL-3, IL-5, GM-CSF) and leads to the release of preformed e.g. histamine and newly synthesised e.g. LTC₄ mediators. Human basophil reactivity is widely used in allergy diagnosis. It has been studied by examining changes in the staining patterns of the intracytoplasmic granules using toluidine blue, alcian blue or astra blue (basophil degranulation test) [3, 6–10] or by measuring histamine release [11, 12]. These techniques require a high level of expertise and are not generally available in routine clinical immunology or allergy laboratories. More recently, the study of allergen-induced leukotriene release has also been suggested for routine allergy testing [5, 13, 14]. Flow cytometry is a popular method of choice for analysis in clinical immunology and methods based on the staining properties of basophils with alcian blue have been described [7]. Human basophils, in common with other white blood cells express many different surface markers including (IgE, high affinity IgE receptor (Fc epsilon RI), class I HLA antigen, CD11, CD13, CD18, CD26, CD31, CDW32 (Fc gamma RII), CD 33, CD40, CD43, CD44, CD45, CD49d/CD29, CD54) [15–21].

These markers (IgE, CD45, CD18 and CD63) [9, 15, 16, 18, 21–24] have also been used for the flow cytometric detection of basophil activation. Recently, it has been reported that the surface marker CD63 is expressed with a high density on activated basophils but only weakly expressed on resting basophils [21, 23]. The aim of this study was therefore to characterise a two colour flow cytometric method using anti-IgE fluorescein isothiocyanate (FITC) and anti-CD63 phycoerythrin (PE) to analyse human basophil activation.

Introduction

Basophils are important pro-inflammatory cells involved in immediate hypersensitivity and other disease states [1–5].

Materials and methods

Blood sampling and volunteers

Blood samples were taken into heparin (5 IU/ml) and EDTA (5 mM). The subjects were blood donors with a proven basophil response to anti-IgE

and patients who have given their informed consent to participate in the study and allergic to grass pollen with a positive history, positive skin tests and positive specific IgE (>class 3, using CAP-radioimmunoassay (RIA), Pharmacia Upjohn, Uppsala, Sweden).

Anti-IgE induced basophil activation

This method has previously been described in detail [3, 4, 10, 24]. In brief, leukocyte rich plasma was obtained by centrifugation (500 g, 10 min, 4 °C) of 10 ml of whole blood taken on heparin (5IU/ml), EDTA (5 mM). The buffy coat (300 µl) was washed once with a HEPES calcium buffer (HCB): HEPES 20 mM, NaCl 130 mM, KCl 5 mM, Ca^{++} 2 mM, Mg^{++} 1 mM, BSA 1 mg/ml pH 7.4) primed by the addition of 1.5 ml of interleukin-3 (IL-3) (2 ng/ml final concentration) diluted in the same

buffer and preincubated for 10 min at 37 °C. The addition of IL-3 to the incubation medium leads to a preactivation or priming of human basophils and therefore to an increase sensitivity to agonists [13, 25]. The leukocyte suspension (30 µl) was then mixed in microtitre plates with an anti-IgE dilution (30 µl, final concentration 0.2 µg/ml). In all experiments an anti-IgE free control was included by mixing the cells with HCB (30 µl). The plates were then incubated at 37 °C for 30 min.

Analysis of basophil activation by flow cytometry and histamine release

At the end of the incubation period, hepes-EDTA buffer (HEB): HEPES 20 mM, NaCl 130 mM, KCl 5 mM, EDTA-Na3 2 mM, BSA 1 mg/ml pH 7.4) (40 µl) was added to each well and the plates were centrifuged

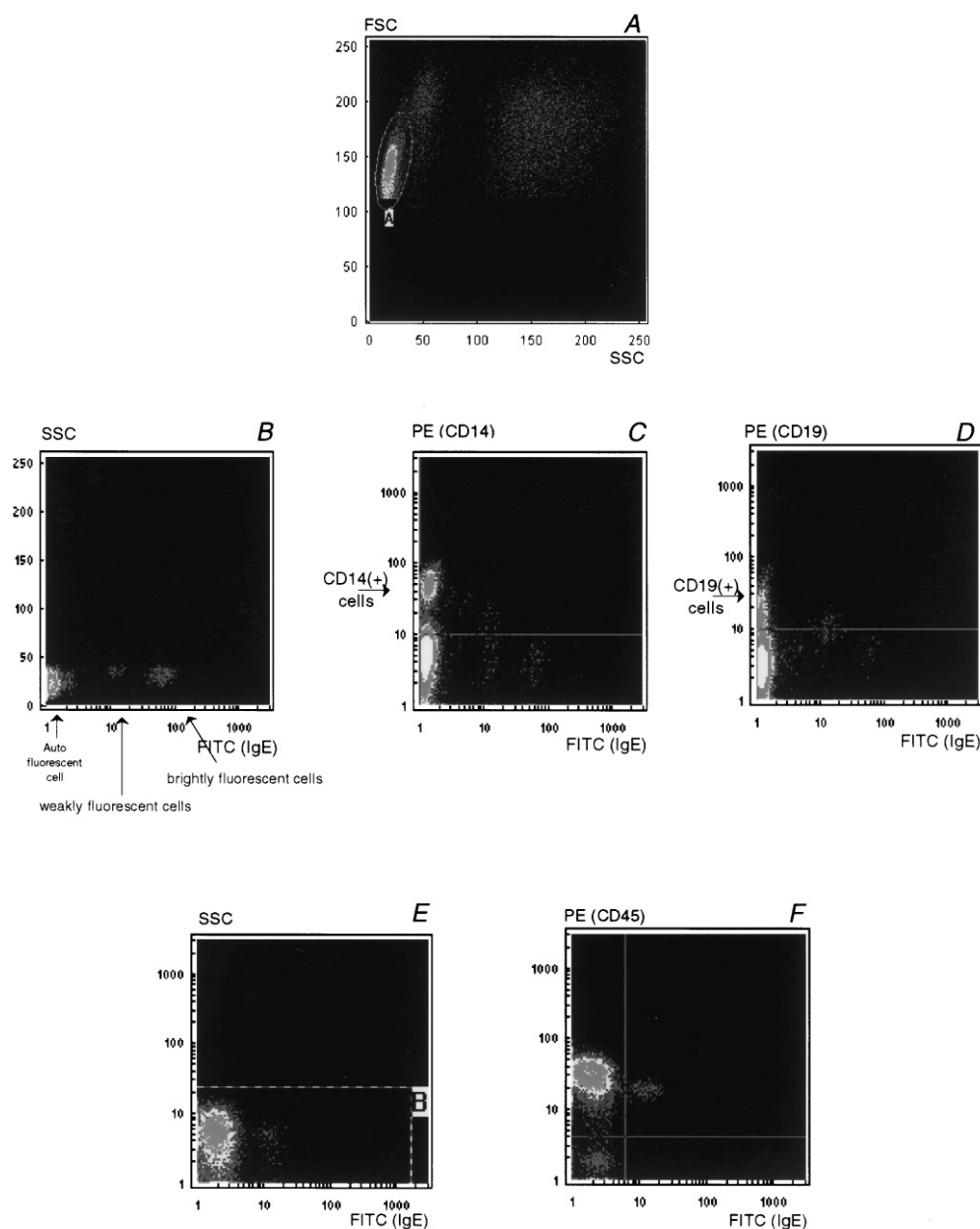


Fig. 1. Cells gated in the region A (A) were labelled according to different procedures: anti-IgE FITC alone (B), anti-IgE FITC and anti-CD14 PE (C), anti-IgE FITC and anti-CD19 PE (D). Cells were analysed by adjusting the FITC photomultiplier at a high gain in order to visualize the weakly fluorescent cells. Using a lower gain for the FITC photomultiplier, these cells were also labelled by anti-IgE FITC alone (E) and by a mixture of anti-IgE FITC and anti-CD45 PE (F).

(500 g, 5 min, 4 °C). Supernatants (40 µl) were discarded or stored at -20 °C until histamine analysis (Immunotech, Luminy, France RIA, sensitivity 0.2 nM with a negligible cross reaction with histamine metabolites and histidine). For flow cytometric analysis, the cell suspensions (20 µl) were mixed with diluted (1:20) fluorescent labelled antibodies (20 µl). The suspensions were incubated for 20 min at room temperature and remaining erythrocytes were lysed by a 3 min incubation with a lysing buffer (3 ml, Ortho Diagnostics, Rochester, USA). The samples were then centrifuged (500 g, 10 min, 4 °C), washed with HEB and the volume adjusted to 250 µl with HEB. Flow cytometric analysis of the labelled cells was performed with a laser tri-colour flow cytometer (Cytoron Absolute, Ortho Diagnostics). Basophils were gated in the lymphocyte region of the forward scatter (FSC) which relates to the cell size and side scatter region (SSC), which relates to the cell's granularity and the fluorescence intensity analysed. Percentage activation (CD 63+, anti-IgE FITC bright cells/anti-IgE FITC bright cells × 100) was calculated by the cytometer.

Specificity of anti-IgE labelling

In order to examine the phenotype of the anti-IgE positive populations, the same suspensions were labelled with anti-IgE FITC and a second antibody. These were: anti CD14 PE (which is expressed on cells of the monocyte/macrophage lineage); anti-CD19 PE (which is expressed on all normal B cells) and anti CD45 PE (which is expressed on all human leukocytes).

Comparison of flow cytometry and histamine release

Two set of experiments were performed: i) Comparison of the sensitivities of the two methods. A leukocyte suspension was passively sensitized, in parallel, by 6 sera having grass pollen specific IgE levels ranging from 4.3 to 33 kU/l. This method described previously [10] consists in a stripping of the bound IgE by a short incubation at low pH and an incubation (90 min, 37 °C) with the tested sera. Leukocyte

suspensions were then tested as described above. ii) Quantitative comparison of the two methods. A leukocyte suspension was passively sensitized with, in parallel, 5 sera having grass pollen specific IgE levels ranging from 29 to 49 kU/l. In both cases, cell pellets were used for flow cytometry and supernatants for histamine release. Basophil activation was induced by a target anti-IgE concentration (0.2 µg/ml) and two allergen extract concentrations (10^{-2} and 10^{-3} of the glycerinated extract used for prick tests).

Specificity of the CD63 labelling

To examine the specificity of the anti-CD63 labelling, cell suspensions were activated with anti-IgE (0.2 ng/ml) and incubated with anti-CD63 FITC antibody in the presence of anti-CD14 PE or anti-CD19 PE.

Isotype controls

Cell suspensions were activated with anti-IgE (0.2 ng/ml) and incubated with goat IgG FITC (anti-IgE FITC isotype control) and mouse IgG₁ PE (anti-CD63 isotype control).

Interference by platelets

Cell suspensions (with and without anti-IgE activation) were labelled with anti-CD41 FITC (which recognises the antigen gpIb expressed by platelets and megakaryocytes) and anti-CD45 PE, anti-CD19 PE, a mixture of anti-CD4 PE and anti-CD8 PE, anti-IgE PE or anti-CD63 PE.

Reagents

Hepes, NaCl, KCl, glucose, BSA, heparin, EDTA Na₃ were purchased from Sigma (St. Louis, MO, USA). Mab anti-IgE FITC, anti-CD45 PE,

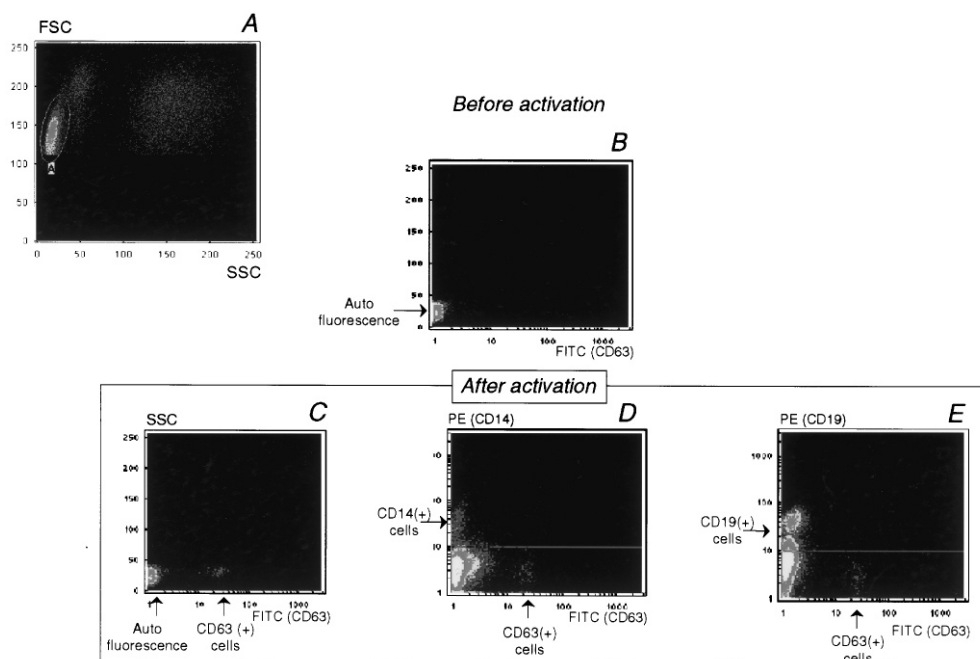


Fig. 2. Cells gated in the region A (A) were labelled by an anti CD63 FITC before (B) and after grass pollen extract induced basophil activation (C, D, E). Activated cells were also labelled by an anti-CD14 PE (D) or an anti-CD19 PE (E).

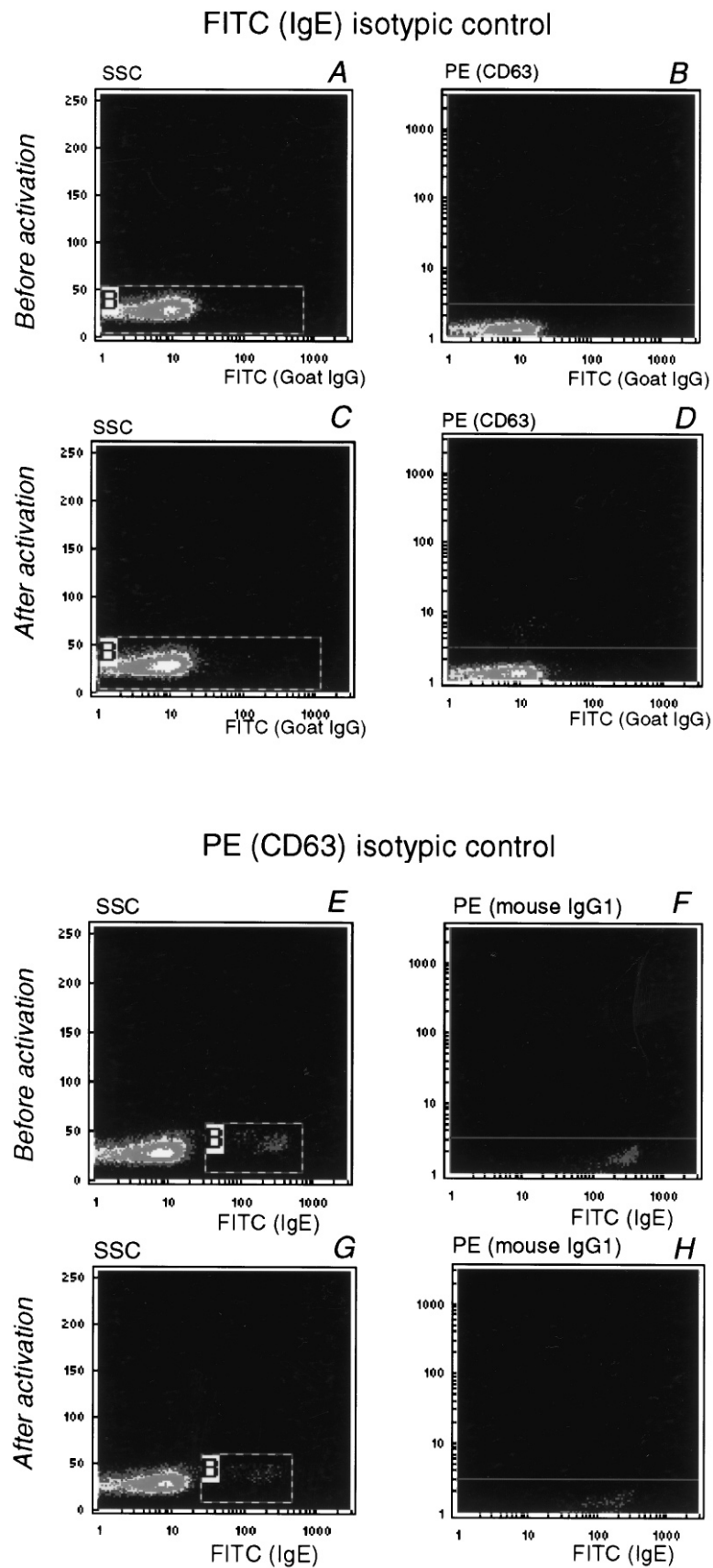


Fig. 3. Labelling specificity was checked by two isotypic controls: A goat IgG FITC (anti-IgE isotypic control) before (A, B) and after (C, D) basophil activation and a mouse IgG1 PE (anti-CD63 isotypic control) before (E, F) and after (G, H) basophil activation. Basophil activation was induced by a grass pollen extract. Blood was taken from a pollen allergic patient.

anti-CD 63 PE, anti-CD14 PE, anti-CD19 PE, anti-CD41 FITC and avidin PE were purchased from Tebu (Le Perray en Yvelines, France). Anti-IgE biotin was purchased from Bio-Sys (Compiègne, France). Grass pollen extract (glycerinated extracts as used in prick tests) was purchased from Dome Hollister Stier, Epernon, France). All polystyrene material was disposable (CML, Nemours, France).

Statistical analysis

The statistical analysis was performed with the Instat statistical software (Sigma laboratory). The correlation between flow cytometry and histamine release was calculated by the Wilcoxon distribution free paired test.

Results

Specificity of anti-IgE labelling

Human basophils are found in the lymphocyte region of the SSC/FSC pattern. This region was gated (Fig. 1A). In this region, 3 populations could be differentiated by their mean intensity of fluorescence (MIF) for anti-IgE FITC: auto-fluorescent, weakly fluorescent and brightly fluorescent (Fig. 1B). The majority of the CD14 positive and CD19 positive cells were found above the auto-fluorescent population (Fig. 1C and D). However, some were found

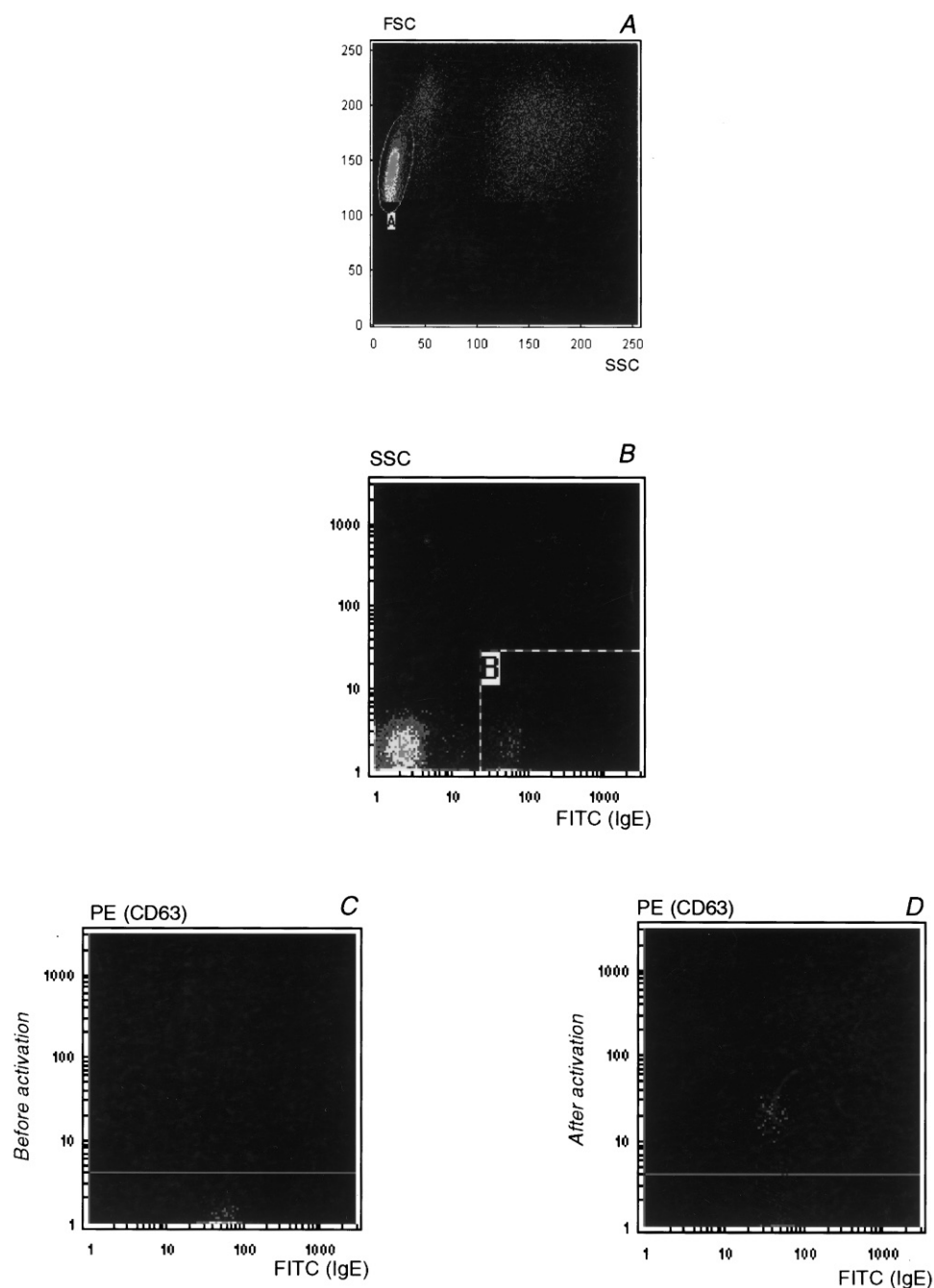


Fig. 4. The whole protocol used for the flow cytometric analysis of basophil activation was based on gating the cells situated in region A (A), followed by selection of the anti-IgE FITC bright cells (B) and calculation of percentage of CD63 positive cells before (C) and after (D) basophil activation, here induced by a grass pollen extract. The electronic thresholds were set according to the usual position of the different cell populations.

Table 1. Statistical analysis of percentage activation measured by flow cytometry and related to 6 experiments for which the same test was repeated 24 times on the same leukocyte suspension. Basophil activation was induced by 0.2 µg/ml of anti-IgE.

Experiment no	Mean percentage activation	Standard deviation	Variation coefficient (%)
1	8.9	2.2	25
2	11.7	2.8	23
3	25.6	3.9	15
4	32	3.7	11.8
5	43.1	2.9	6.4
6	46.4	2.9	6.3

above the weakly fluorescent population (IgE bearing B lymphocytes and monocytes). No CD14 positive or CD19 positive cells were observed above the brightly fluorescent population. Labelling of the same cell suspension with anti-IgE FITC and anti-CD45 PE demonstrated that all anti-IgE brightly fluorescent cells expressed the pan-leukocyte CD45 antigen (Fig. 1E and F).

Specificity of the CD63 labelling

After cell activation with anti-IgE neither CD14 nor CD19 positive cells were observed above the CD63 positive population (Fig. 2).

Isotype controls

No FITC positive cells were detected after labelling with goat IgG FITC (Fig. 3A and B). After activation, CD63

positive cells were located on top of the auto-fluorescent cell population (Fig. 3D). Similarly after labelling with mouse IgG₁ PE, no PE positive cells were detected after activation (Fig. 3E-H).

Interference by platelets

The use of high gain on the photomultiplier allowed the differentiation of three cell populations within the cells gated in the lymphocyte region of the SSC/FSC pattern: auto-fluorescent, weakly fluorescent (intermediate) and brightly fluorescent. The intermediate population comprised of leukocytes (100% CD45 positive). These were mainly CD4 and CD8 positive lymphocytes (51%) and B lymphocytes (18%). A similar percentage of these cells was found in the auto-fluorescent region (50.9 and 15.1%, respectively). Bright anti-IgE PE cells were found above the auto-fluorescent (1.2%) and intermediate cells (1.1%). No CD63 positive cells were detected before activation with anti-IgE above the auto-fluorescent (0.04%) or intermediate cells (0%). After activation they were detected mainly above the intermediate cells (0.07% and 0.47% respectively). Neither IgE positive nor CD63 positive cells were detected above the platelet population.

Analysis of basophil activation by flow cytometry

The flow cytometric method was based on 3 steps: firstly lymphocyte population gating (Fig. 4A), secondly gating of brightly fluorescent FITC (IgE positive) cells (Fig. 4B) and finally calculation of the percentage brightly fluorescent PE cells (CD63 positive) by the cytometer before (Fig. 4C)

Table 2. Histamine release (HR) and basophil activation measured by flow cytometry (BAT) were compared after passive sensitization of leukocytes suspensions by sera having different grass pollen specific IgE levels. Basophil activation was induced by 0.2 µg/ml of anti-IgE (experiments A and B) and 10⁻² (experiment A) and 10⁻² (1) and 10⁻³ (2) (experiment B) of the grass pollen extract.

		Grass pollen specific IgE level (kU/l)					
Experiment A		4.4	13	16	20	25	33
Controls (%)	HR	1.7	3.6	2.6	0.9	6.4	4.6
	BAT	0.7	3.1	3.9	3.4	5.4	2.3
Anti-IgE (%)	HR	3.9	4.5	2.1	18	19.7	25.4
	BAT	19.3	24.4	20.5	22.7	19.8	17.1
Allergen (%)	HR	5.1	7.7	5.7	5.4	35.8	48
	BAT	20.5	50.2	34	39.5	36.7	39
Experiment B		29	30	33	45	49	mean ± sd
Controls	HR	1.9	1.8	1.9	1.9	1.9	1.89 ± 0.04
	BAT	2.4	6.7	6.9	4.3	6	5.3 ± 1.9
Anti-IgE	HR	30.1	34.6	41.6	38	46.8	38.2 ± 6.4
	BAT	25.9	38.5	28.8	35.7	33.7	32.5 ± 5.1
Allergen	HR (1)	39.1	58.3	52.6	55.7	51	51.4 ± 7.4
	BAT (1)	62.9	69.3	45.9	55.7	39.7	54.7 ± 12.1
Allergen	HR (2)	19.8	48.5	53.8	54.5	59.6	47.2 ± 15.8
	BAT (2)	37	49	45.7	55.1	42.6	45.9 ± 6.8

Results are expressed in percentage release or activation and mean results are expressed in percentage ± SD, spontaneous release or activation being deduced. NS = not significant (Wilcoxon paired test).

and after (Fig. 4D) activation with anti-IgE. In a series of repeated measurements, 24 samples of the same leukocyte suspension were analysed by flow cytometry as described above. The statistical data related to a series of six experiments, percentage activation ranging from 8.9 to 46.4%, are represented in Table 1. According to these statistical data, the positivity threshold was set at 5% over the spontaneous percentage of activation for two successive agonist concentrations or at 10% for a given agonist concentration.

Comparison of flow cytometry and histamine release.

A significant histamine release (6% higher than the spontaneous release) was observed after passive sensitisation for grass pollen specific IgE levels higher than 20 kU/l, basophil activation being induced by an anti-IgE or by the grass pollen extract. A significant activation was observed by flow cytometry for the whole range of specific IgE levels (Table 2).

For specific IgE levels higher than 29 kU/l no significant difference was observed between the mean results obtained by the two methods for the agonist free controls and for the tests performed in the presence of anti-IgE and grass pollen extract.

Discussion

The CD63 antigen is a 53 kD glycoprotein mainly found on the membrane of activated platelets and activated basophils. In human basophils, CD63 protein is present on the stroma of intracytoplasmic granules.

After activation, CD63 is expressed on the basophil surface. Using flow cytometry, populations of CD63 positive and negative cells are easily separated. Thus basophil activation can be readily assessed by calculating the percentage of anti-IgE positive cells which are also CD63 positive. The contribution by other IgE bearing cells is low, as IgE bound to low affinity receptors will be washed away during the successive incubations in buffer and only cells with a high density of IgE epitopes will be selected on the flow cytometer. The control studies described in this paper demonstrate that cells gated in the lymphocyte region are B and T lymphocytes, neutral killer (NK) cells, some monocytes, platelets and basophils. Those expressing a high density of IgE epitopes had the phenotype IgE positive, CD14 negative, CD19 negative, CD41 negative or +/-, CD45 positive and CD63 negative or positive and hence were basophils. The CD41 +/- cells are probably basophils bearing platelets as this population was CD45 positive but CD63 expression is not the result of IgE dependent platelet aggregation as concluded by other authors [19,20] since isolated platelets (high MIF cells) did not express the CD63 antigen after anti-IgE activation and since CD63 is expressed by purified basophils [21]. Furthermore, no significant quantitative difference was found between histamine release and percentage CD63 positive cells after grass pollen extract or anti-IgE induced basophil activation. Platelets have been observed on the surface of basophils by electron microscopy. A non-specific binding of platelets to different cell types is suggested since a similar proportion of T and B lymphocytes

was found in the auto-fluorescent and intermediate cell populations.

If we consider the different measurable steps of basophil activation (calcium influx, leukotriene release, expression of membrane markers and final release of pre-formed mediators such as histamine), it may be hypothesised that each step is characterised by a triggering threshold in terms of intensity of the activation signal required depending on the concentration and the potency of the agonist and on the presence of priming agents, such as IL-3, within the incubation medium. This may explain the discrepancies observed between various methods used for measuring basophil activation.

Flow cytometry may represent a new fast and sensitive method for allergy diagnosis and for pharmacological studies of basophil activation.

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