

Flow Cytometric Evaluation of Human Basophils¹

Pierre Gane², Catherine Pecquet, Patrick Lambin, Nissen Abuaf, Francisque Leynadier, and Philippe Rouger

Institut National de Transfusion Sanguine (P.G., C.P., P.L., P.R.); Centre d'Allergie, Hôpital Rothschild (C.P., F.L.); Laboratoire d'Hématologie et Immunologie Biologique, Hôpital Rothschild (C.P., N.A.), Paris, France

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The expression of CD45 and IgE cell surface antigens on human leucocytes was studied by flow cytometry. More than 80% of sorted cells that expressed low CD45 (CD45^{dim}) and high IgE (IgE^{bright}) antigen site density were identified as basophils. Immunomagnetic depletion of the CD45^{dim}-IgE^{bright} cell subset by a biotin-coupled anti-IgE antibody and streptavidin-coated magnetic beads was greater than 90%, and more than 80% of cells binding significant numbers of beads exhibited the morphological char-

acteristics of basophils. Interestingly, when the cell staining was performed in the presence of calcium and magnesium, we observed a significant increase of CD45 and an equivalent decrease of IgE cell surface expression, as well as an IgE concentration dependent diminution of the number of CD45^{dim}-IgE^{bright} cells.

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Studies on human basophils have provided important information on their role in allergic reactions (7,8). The binding of allergens to IgE antibody fixed on the cell surface of basophils activates them, leading to degranulation and the secretion of a number of chemical mediators, including histamine. Activation and degranulation can also be induced by the reaction of anti-IgE antibodies with cell-surface IgE (5,14). Basophil activation/degranulation has been quantified by the measurement of secreted histamine (7) and by direct microscopic examination (6). The principal advantage of the latter method is its simplicity. However, the low frequency of basophils in the human peripheral blood leucocyte (PBL) population—they comprise 1% or less of circulating lymphocytes—makes that method rather unreliable.

Flow cytometry is now widely used to identify and quantify cell surface antigens as well as subpopulations of cells expressing these antigens. Human basophils express IgE, CD11, CD13, CD18, CD26, CD31, CDw32, CD33, CD40, CD43, CD44, CD45, CD54, and Leu8 on their surface (1,2,3,16,17). Nevertheless, none of these antigens is specific for basophils. Using a combination of anti-IgE and CD45 antibodies, we have developed a 2-color immunofluorescence procedure for the cytometric evaluation of human basophils which may facilitate further functional studies of these cells.

MATERIALS AND METHODS

Cell Preparation

Peripheral blood from 7 nonallergic and 22 allergic adult volunteers was collected on EDTA. The blood was diluted 1:2 in phosphate buffered saline (PBS, pH 7.2, BioMerieux, Marcy-L'Etoile, France) and layered over MSL (density, 1.077; Eurobio Laboratories, Paris, France), and centrifuged at 300 × g for 30 min at room temperature. The leucocyte-rich layer was recovered and washed in PBS. Alternatively, leucocytes were prepared by lysing the red blood cells with a formaldehyde-free lysing buffer (Ortho D.S., Raritan, NJ) (11). The white blood cells were then washed in PBS and stored at room temperature until they were labelled.

Antibodies

The following antibodies were used: fluorescein-conjugated HLe-1 (CD45-FITC), Leu-11a (CD16-FITC), Leu-16 (CD20-FITC) monoclonal antibodies (MAbs)

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²Address reprint requests to P. Gane, Institut National de Transfusion Sanguine, 6, rue Alexandre Cabanel, 75739 Paris Cedex 15, France.

(Becton-Dickinson, Mountain View, CA); and biotinylated goat anti-human IgE (Vector Laboratories, Burlingame, CA). R-phycoerythrin-conjugated streptavidin was obtained from Vector Laboratories.

Immunofluorescence

Staining was performed at room temperature ($20 \pm 2^\circ\text{C}$). PBS or PBS supplemented with 4.6 mM CaCl_2 and 2 mM MgCl_2 (PBS/Ca/Mg) were used for the cell suspension and for the dilution of anti-IgE and CD45 antibodies. Antibodies were added to $1\text{--}3 \times 10^6$ cells for 20 min. After washing, the cells were resuspended in 1 ml of PBS or, alternatively, were stained with 0.5 μg of R-phycoerythrin-conjugated streptavidin for 20 min at 4°C .

Cytometric Analysis

We used an Ortho Spectrum III cytometer interfaced to a 2140 analyser (Ortho) and a fluorescence-compensated dual logarithmic amplifier module. Elimination of debris and gating of cell populations were performed using their forward and side scatter characteristics. Fluorescence signals were subjected to logarithmic amplification (3.5 decades). An Ortho 50H flow cytometer was used for cell-sorting experiments.

Immunomagnetic Isolation of IgE Bearing Cells and Analysis of the Depletion of the Basophil Subset

Streptavidin-coated magnetic beads (Dynabeads M-280 Streptavidin, Dynal, Oslo, Norway) were used according to manufacturer's instructions. Briefly, 0.2 μg of the biotinylated anti-IgE antibody were added to 2×10^6 mononucleated cells from nonallergic donors. After washing, Dynabeads were added to the cell sample. Different bead-to-cell ratios, ranging from 3:1 to 100:1, were used. A preparation in which the anti-IgE antibody was omitted served as the control. Smears of positive rosetted cells isolated with a magnet (magnetic particle concentrator, MPC, Dynal) were then prepared using a cytocentrifuge, and the cells were stained with May-Grünwald-Giemsa. The nonrosetted cells were reacted with 20 μl of the CD45-FITC MAb and 0.5 μg of R-phycoerythrin-conjugated streptavidin for 20 min. In the control preparation, the magnetic beads were omitted.

Statistical Analysis

Results are given as mean values $\pm$ s.e.m. Inter-group statistical analysis was performed using the unpaired Student's *t* test.

RESULTS

Flow Cytometric Analysis of CD45 and IgE Expression on Peripheral Blood Leucocytes

CD45 is expressed on all the leucocytes isolated on a density gradient or by the blood lysis technique (Fig. 1B). As can also be seen in Figure 1B, a discrete dimly stained (CD45^{dim}) subpopulation (region 6), which ac-

counts for 0.2–3% of the total leucocytes, was always observed with HLe-1 anti-CD45 MAb. The CD45 mean fluorescence intensity (MFI) of this cell subset was very similar to that of polymorphonuclear cells.

On the other hand, a subset containing an equivalent percentage of cells expressed a high density of IgE ($\text{IgE}^{\text{bright}}$) surface antigens (Fig. 1C, region 6). The MFI of this subset was highly variable, ranging from 30 to 180 (linear scale on 200 channel histograms). In some samples, a cell population with a lower MFI (IgE^{dim}) could also be seen (Fig. 1C, region 5). Most of these cells, which were found to be $\text{CD45}^{\text{bright}}$ leucocytes, were identified as monocytes.

Study of the IgE expression on CD45^{dim} cells and of CD45 expression on $\text{IgE}^{\text{bright}}$ cells, as well as 2-color analysis (Fig. 1D), clearly demonstrated that the vast majority of CD45^{dim} cells were also $\text{IgE}^{\text{bright}}$ and that all $\text{IgE}^{\text{bright}}$ cells were CD45^{dim} .

As shown in Table 1, $\text{CD45}^{\text{dim}}\text{-IgE}^{\text{bright}}$ cells were found in the region of lymphocytes (Fig. 1A, region 1) and in another region located between the lymphocytes and monocytes (Fig. 1A, region 2). Interestingly, the polymorphonuclear region was constantly devoid of the $\text{CD45}^{\text{dim}}\text{-IgE}^{\text{bright}}$ subset, indicating that cells isolated by density gradient separation ($d = 1.077$) can be used for the determination of $\text{CD45}^{\text{dim}}\text{-IgE}^{\text{bright}}$ cells. Examination of the expression of the CD20 B cell antigen and of the large granular lymphocyte CD16 antigen on leucocytes from 2 allergic and 2 nonallergic individuals showed that less than 0.1% of mononucleated cells co-expressed IgE and CD16 or IgE and CD20 (data not shown).

Using the polyclonal biotinylated anti-human IgE antibody (400 ng/assay) and the HLe-1 anti-CD45 MAb (20 μl /assay), the percentage of $\text{CD45}^{\text{dim}}\text{-IgE}^{\text{bright}}$ cells was $1.2 \pm 0.25\%$ and $1.7 \pm 0.10\%$ of density gradient-isolated mononuclear cells from nonallergic and allergic individuals, respectively ($P = 0.33$).

Cytochemical Characterization of the $\text{CD45}^{\text{dim}}\text{-IgE}^{\text{bright}}$ Cells

More than 80% of the cells from a nonallergic donor, expressing the $\text{CD45}^{\text{dim}}\text{-IgE}^{\text{bright}}$ phenotype and isolated by sorting, were identified as basophils on microscopic examination. To study the possibility of a rapid basophil isolation procedure, we used an immunomagnetic technique, employing a biotinylated anti-IgE antibody and streptavidin-coated magnetic beads. The positive selection of IgE binding cells and their cytochemical characterization were best achieved with the lowest bead-to-cell ratios (3:1 to 10:1). The use of higher proportions of beads made microscopic examination of the cells very difficult due to the large number of particles (up to 30) attached to each cell and to randomly dispersed free beads. Cell preparations obtained after immunomagnetic selection of IgE bearing cells were contaminated with monocytes and a very few lymphocytes. Nevertheless, more than 90% of the non-basophils did not bind any particles, whereas more

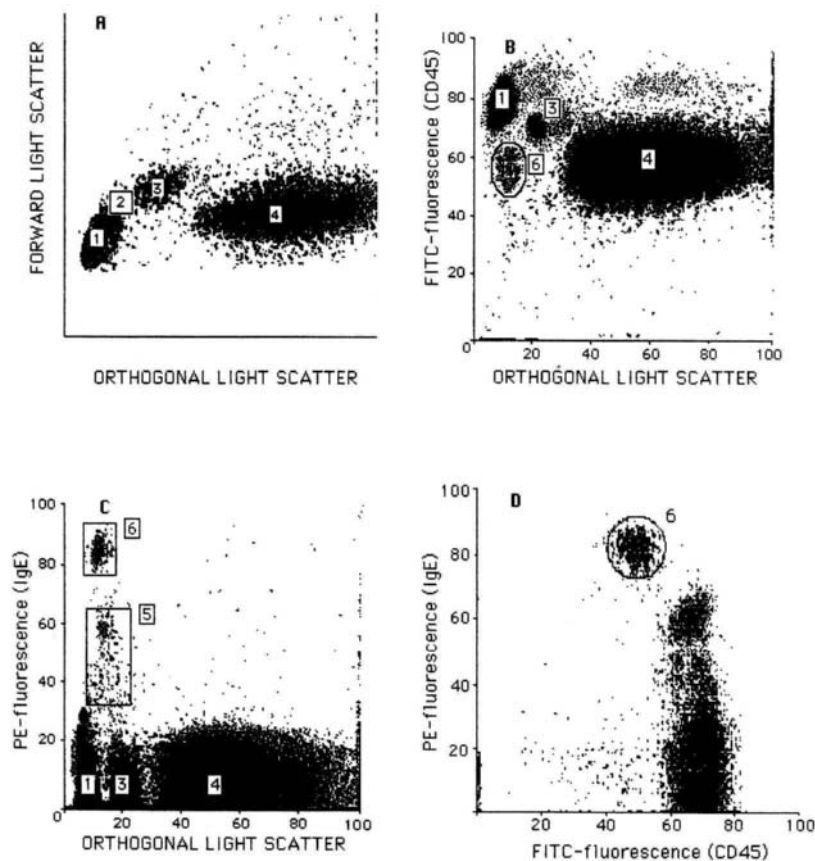


FIG. 1. Peripheral lymphocytes from a nonallergic individual, isolated after lysis of red blood cells, stained with Hle-1 anti-CD45 MAb and the biotinylated anti-human IgE. **A:** Correlative display of orthogonal light-scatter (X axis) vs. forward light-scatter (Y axis). Region 1: lymphocytes, region 2: "large lymphocytes" region (see text), region 3: monocytes, region 4: neutrophils. **B, C:** Correlative display of orthogonal light-scatter (X axis) vs. CD45 (**B**) and IgE (**C**) (Y axis).

In **B** it can be seen that neutrophils (region 4) and CD45^{dim} cells gated in region 6 display very similar CD45 mean fluorescence intensities (MFI). Figure **C** shows the IgE^{bright} subset (region 6) as well as a cell population with a lower IgE MFI (region 5). Cells gated in region 6 in **B** and **C** display intermediate orthogonal light-scatter properties between lymphocytes and monocytes. **D:** Correlative display of CD45 (X axis) vs. IgE (Y axis). CD45^{dim}-IgE^{bright} cells are gated in region 6.

than 80% of the cells that bound 4 or more beads were identified as basophils (Fig. 2). Control preparations, in which the anti-IgE antibody was omitted, were also notably enriched with monocytes in comparison with the starting density-gradient isolated cells (data not shown). The depletion of the CD45^{dim}-IgE^{bright} cell population varied from 43% to 90%, with bead-to-cell ratios ranging from 3:1 to 100:1.

Cell Staining in the Presence of Calcium and Magnesium

In the 29 samples from allergic and nonallergic donors, an increase in basophil CD45 MFI (up to 50%) and an equivalent reduction in surface IgE MFI was observed in the presence of calcium and magnesium, as compared with cells reacted without these cations. The incubation of cells in PBS/Ca/Mg alone (without anti-IgE antibody) did not increase CD45 MFI (data not shown). As shown in Figure 3A and 3B, anti-IgE-induced CD45 and IgE surface antigen modulation was

clearly dose dependent. Interestingly, the modulation of CD45 expression was observed both with high and low anti-IgE concentrations (Fig. 3B), whereas a reduction in IgE MIF was only observed with the highest anti-IgE concentrations (Fig. 3A).

In addition, an anti-IgE dose-dependent reduction in the number of CD45^{dim}-IgE^{bright} cells could also be noticed (Fig. 3C). Using the polyclonal anti-IgE antibody at the concentration of 400 ng/assay, the decrease in the number of CD45^{dim}-IgE^{bright} cells ranged from 0% to 70%. However, no significant difference was observed between allergic (mean $29 \pm 4.9\%$) and nonallergic (mean $21 \pm 4.4\%$) individuals ($P = 0.26$).

DISCUSSION

A flow cytometric method for the quantification of basophils and their degranulation that used the measurement of axial light loss has been previously reported (12). We describe here a flow cytometric 2-color immunofluorescence technique for the evaluation of

Table 1
Percentage of CD45^{dim}-IgE^{bright} Cells in Forward and Orthogonal Light-Scatter Defined Leucocyte Populations

Cell population	Region number (Fig. 1A)	Percentage of CD45 ^{dim} -IgE ^{bright} cells
Total leucocytes	1 + 2 + 3 + 4	0.4 ± 0.20*
Lymphocytes	1	2.4 ± 0.21
Monocytes	3	0.4 ± 0.39
Granulocytes	4	0.0
"Large lymphocytes" ^a	2	8.0 ± 1.16
Lymphocytes and monocytes ^b	1 + 2 + 3	2.0 ± 0.35

*Mean ± SEM of 4 experiments.

^aThe "large lymphocytes" region includes the largest lymphocytes as well as the intermediate region between lymphocytes and monocytes (Fig. 1, region 2).

^bThe region of "lymphocytes and monocytes" includes lymphocytes, monocytes, and the "large lymphocytes" region.

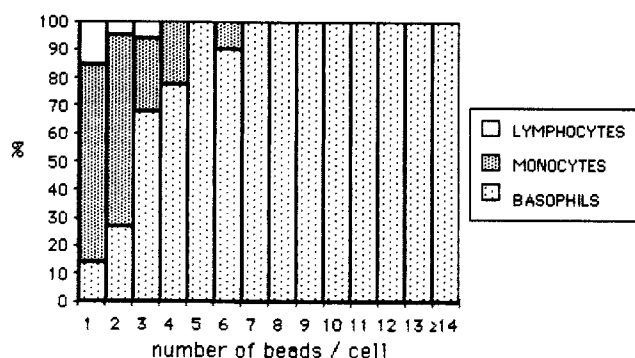


FIG. 2. Repartition of basophils, monocytes, and lymphocytes in the cell population obtained by immunomagnetic selection of IgE bearing cells as a function of the number of beads attached to the cells. Mononuclear cell from a nonallergic donor were added with the biotinylated anti-human IgE and after washing, with streptavidin-coated magnetic M-280 Dynabeads (bead to cell ratio, 10:1). Positive rosetted cells attracted by the magnet were washed, cytocentrifuged, and then stained with May-Grünwald-Giemsa. Microscopic examination of 510 cells.

human basophils. Basophils are known to express surface IgE and CD45, the latter at low density (10). However, to our knowledge, the CD45^{dim}-IgE^{bright} double phenotype has never been used to evaluate these cells. In the present study, cell-sorting experiments and immunomagnetic isolation of IgE^{bright} cells demonstrated that basophils alone express this phenotype. Contamination by monocytes of cell preparations obtained by immunomagnetic isolation of IgE bearing leucocytes might be explained by the expression of low-affinity IgE-binding sites (CD23) on monocytes (4). In addition, CD23 may also mediate the adhesion and phagocytosis of IgE-coated particles (15). Consequently, immunomagnetic separation of IgE bearing cells seems to be a useful and rapid technique to obtain basophil enriched cell preparations but is probably not a suitable method to obtain pure basophil cell preparations.

In previous flow cytometric studies, FITC-IgE anti-DNP (13) and Bsp-1 (3) MAbs were used for the isolation of basophils and their phenotypic analysis. To our knowledge these antibodies are unfortunately not available for general use. Anti-IgE antibodies alone have also been used for this purpose (1). In our opinion, however, 2-color immunofluorescence in cytometric analysis of basophils seems to be critical because of the low frequency of these cells. Data obtained in our study showed that the evaluation of basophils based on either their IgE cell surface expression or their low CD45 antigen site density alone leads to an overestimation of their number, ranging from 3% to 25% and 15% to 70%, as compared with counts of CD45^{dim}-IgE^{bright} cells. Moreover, the use of the CD45 MAb allows the exclusion of IgE-reactive lymphocytes, monocytes, and red blood cells, as well as most of the cell debris, the presence of which was found to be one of the major causes of the underestimation of basophils in flow cytometry (2). It is worth noting that overexpression of CD23 on B cells, monocytes, and CD16 NK lymphocytes has been observed in atopic patients (15).

Anti-IgE antibodies have been reported to alter the expression of surface IgE and CD45 (2), as well as CD63 (9) and Leu-8 (2) expression on basophils. In the present work, we confirm these findings. In addition, we show a dramatic IgE dose-dependent decrease of the number of detectable CD45^{dim}-IgE^{bright} cells in the presence of calcium and magnesium. A quenching phenomenon due to the addition of increasing amounts of anti-IgE cannot be excluded. However, since anti-IgE antibodies were found to activate and degranulate basophils in the presence of these cations (2,5,12,14), this observation may also be explained by the bridging and the internalization of basophil cell surface IgE. Thus, the decrease in the number of detectable basophils we observed may reflect the activation of these cells.

Incubation of cells with anti-IgE and CD45 antibodies at 37°C amplifies the observed modulation of IgE and CD45 expression, notably that of CD45 (data not shown). However, the temperature of incubation of 20°C was chosen because blood samples and cell preparations were kept at room temperature and, in preliminary experiments, we had observed that important variations in incubation temperature might induce basophil activation and degranulation.

Studies on allergen-induced basophil activation based on changes in surface IgE and CD45 antigens detected by flow cytometry are now in progress in our laboratory (manuscript in preparation). Preliminary results show that specific allergen-induced activation can be clearly demonstrated with cells from allergic individuals. Very low concentrations of anti-IgE antibody that do not induce detectable basophil activation can also be used to label the cells. Thus, the fact that the immunofluorescence procedure used to characterize basophils may itself induce changes in IgE and CD45 surface antigens does not constitute an important disadvantage. It is worth noting that flow cyto-

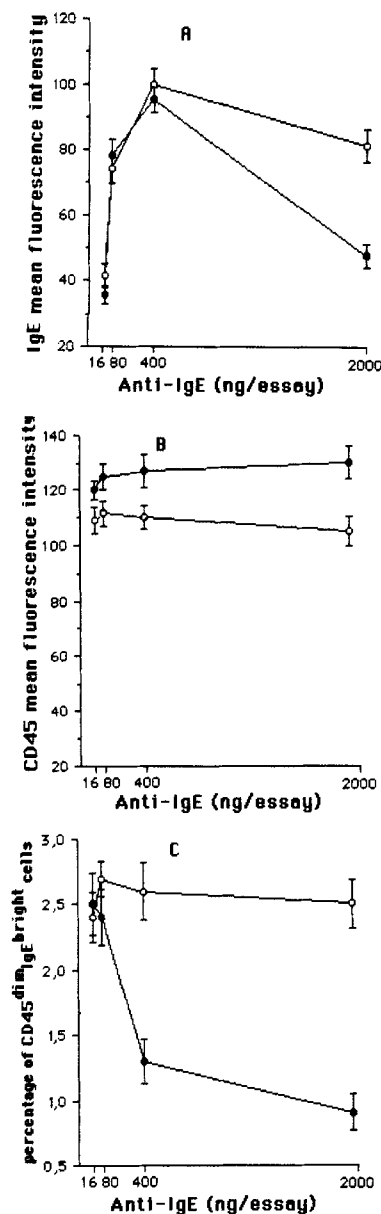


FIG. 3. Concentration-dependent effect of anti-IgE on IgE (A) and CD45 (B) expression on basophils and on the evaluation of the number of CD45^{dim}-IgE^{bright} cells (C) in the absence (open circles) and presence (black circles) of calcium and magnesium. Representative experiment with mononuclear cells from a nonallergic individual. Values represent mean $\pm$ SEM of 2 determinations.

metric analysis of basophil activation is a very rapid method that can be used with samples having very few basophils, which is not the case for microscopic tech-

niques. Our observations may greatly facilitate further studies on the activation of human basophils.

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