

Purification of morphologically and functionally intact human basophils to near homogeneity

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Abstract

Certain studies on basophils require highly purified functionally intact cell preparations. Here, a three-step procedure is described that meets these requirements. The procedure consists of a Ficoll density gradient step, counterflow elutriation and negative selection by magnetic cell sorting (MACS). The mean purity of basophils obtained from 30 donors was $97.6 \pm 3.96\%$ with a viability of $99.6 \pm 0.83\%$. The recovery rate was $49.7 \pm 15.6\%$. The cells had a normal morphological appearance as assessed by May–Grünwald-stained cytopins and were functionally intact as shown by their unaltered capacity to release histamine and interleukin 4 (IL-4) following immunological activation. This procedure is a clear improvement over currently available techniques and should facilitate future investigations on basophils. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Basophils are a small population of granulocytes that account for less than 1% of total leukocytes in

peripheral blood. During the course of allergic reactions, basophils have been shown to migrate into tissues participating in inflammatory reactions (Durham, 1998). Upon crosslinking of high affinity IgE receptors (FcεRI) or in response to other stimuli, basophils rapidly release mediators such as histamine and leukotrienes (Marone, 1988) as well as cytokines such as IL-4 and IL-13 (Brunner et al., 1993; Gibbs et al., 1996). Therefore, basophils may play an important role not only in the effector phase, but also in the initiation of allergic inflammation and parasitic disease.

Studies on basophils have been hampered by the lack of efficient procedures to obtain highly purified,

Abbreviations: BSA, bovine serum albumin; MACS, magnetic cell sorting; IL, interleukin; PFA, paraformaldehyde; IMDM, Iscove's modified Dulbeccos's medium; HBSS, Hank's balanced salt solution without Ca^{2+} and Mg^{2+} ; PBS-BSA, phosphate-buffered saline with 0.5% BSA; Tris-T, Tris with 0.05% Tween 20; PE, phycoerythrin; FITC, fluorescein isothiocyanate; MNCs, mononuclear cells; EDTA, ethylenediaminetetraacetic acid

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functionally intact cells. Contaminating cells can activate basophils by releasing factors such as IL-3, granulocyte–macrophage colony stimulating factor or platelet-activating factor (Columbo et al., 1990). Furthermore, contaminating cells are able to produce identical cytokines or express the same surface receptors as basophils and in this manner interfere with the determination of these parameters for basophils. Therefore, it is essential to obtain highly purified basophils for investigations where interference by other cells must be avoided. Various techniques have been developed for the purification of basophils (Weil et al., 1983; Bodger and Newton, 1987; Schroeder and Hanrahan, 1990; Mul et al., 1992; Tanimoto et al., 1992; Bjerke et al., 1993; Kopley et al., 1994; Willheim et al., 1995; Gibbs et al., 1997). Several techniques provide basophils of purities > 95%. However, these techniques either include positive selection (Weil et al., 1983; Willheim et al., 1995), which may lead to activation or inactivation of basophils, or they result in low yields (Bjerke et al., 1993; Kopley et al., 1994).

Here, we describe a highly efficient technique for the purification of human basophils to almost homogeneity. This method is based on our previous protocols (Falcone et al., 1996; Gibbs et al., 1997) consisting of density gradient centrifugation, counterflow elutriation, and negative selection by immunomagnetic beads. Compared to the previous protocols, the introduction of the MACS system as a third step led to a dramatic increase in the purity of morphologically and functionally intact basophils. Also, the yield was higher than in any previously published protocol with a basophil purity exceeding 95%. By this technique, nearly homogeneous basophil preparations can be efficiently obtained for studies where contaminating cells must be excluded.

2. Materials and methods

2.1. Materials

The following reagents and materials were obtained from the suppliers indicated: Trypan Blue solution, May–Grünwald-Stain, BSA, NaN_3 , bovine

insulin, human transferrin (Sigma, St. Louis, MO); NaHCO_3 , EDTA, MgCl_2 , paraformaldehyde (PFA), perchloric acid (Merck, Darmstadt, FRG); FCS (Boehringer Mannheim, Mannheim, FRG); Iscove's modified Dulbecco's medium (IMDM), penicillin, streptomycin, (Gibco BRL, Paisley, UK); streptavidin-alkaline phosphatase (Jackson Immuno Research, West Grove, PA); *p*-nitrophenylphosphate (Sigma, Deisenhofen, FRG); Tween 20 (Merck–Schuchardt, Hohenbrunn, FRG); NaCl, Tris (Serva, Heidelberg, FRG); Ficoll (Biochrom, Berlin, FRG); Percoll (Pharmacia, Freiburg, FRG); Hank's balanced salt solution without Ca^{2+} and Mg^{2+} (HBSS; PAA, Linz, Austria).

2.2. Purification of basophils

2.2.1. Ficoll density centrifugation

Some 216 ml blood from healthy volunteers who had not been purposely selected for high numbers of basophils in their circulation were drawn into syringes (60 ml) containing 6 ml 0.1 M EDTA solution and gently mixed before the blood was carefully layered on Ficoll. In order to minimise losses of basophils, density gradient centrifugation was performed at 4°C since, at this temperature Ficoll density is higher than at 25°C (1078 g/ml vs. 1077 g/ml). Moreover, performing this enrichment step at 4°C minimises the degree of basophil activation. After centrifugation (25 min, $500 \times g$, 4°C), the interphases were pooled, washed and resuspended in 50 ml ice-cold elutriation buffer (HBSS supplemented with 4.2 mM NaHCO_3 and 0.25% BSA, pH 7.4). Total cell numbers and viability were determined by staining with Trypan Blue solution and counting in a Neubauer chamber. Basophil purity was assessed by investigating 1000 cells on cytopins treated with May–Grünwald stain.

2.2.2. Counterflow elutriation

Elutriation was performed with a J2-21 centrifuge and a JE-6B rotor, equipped with a standard chamber (Beckmann Instruments, Palo Alto, CA). The rotor speed was held constant at 3500 rpm ($1220 \times g$) throughout the whole procedure. Interphase leukocytes were loaded into the chamber, and the

flow rate was progressively increased from 30 to 45 ml/min at intervals of 3 ml/min. Several fractions were collected and concentrated by centrifugation (10 min, $500 \times g$, 4°C). After the evaluation of cell numbers and basophil purity, the basophil-rich fractions were pooled.

2.2.3. Magnetic cell separation (MACS)

Further purification was performed using the MACS basophil isolation kit (Miltenyi Biotec, Bergisch Gladbach, FRG) essentially according to the manufacturer's protocol. After centrifugation (10 min, $500 \times g$, 4°C) of the pooled basophil-rich fractions the supernatant was removed completely and the pellet resuspended in PBS-BSA (PBS supplemented with 0.5% BSA) at a ratio of 1 μl PBS-BSA per 10^6 cells. Subsequently, 0.5 μl IgG-containing blocking reagent and 1 μl hapten-labelled antibody cocktail (anti-CD3, anti-CD7, anti-CD14, anti-CD15, anti-CD16, anti-CD36, anti-CD45RA, anti-HLA-DR) were added per 10^6 cells, mixed well and incubated for 10 min at 8°C . Thereafter, 6.5 μl PBS-BSA and 1 μl anti-hapten micro beads were added per 10^6 cells, mixed well and incubated for 15 min at 8°C . Then the cells were introduced onto an LS^+ column (included in the kit) previously washed with 2×3 ml ice-cold PBS-BSA. The unbound fraction containing the basophils was washed out with 4×3 ml of ice-cold PBS-BSA. Basophil number, purity, and viability were evaluated as above. For further handling, the cells were transferred to medium (IMDM supplemented with 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 5 $\mu\text{g}/\text{ml}$ bovine insulin, 50 $\mu\text{g}/\text{ml}$ human transferrin and 10% FCS).

The whole purification procedure was performed in 3.5 h.

2.3. Histamine release

Aliquots of cells, obtained after each purification step, containing 50,000 basophils in 200 μl medium, were stimulated with various concentrations of polyclonal rabbit anti-human IgE antibodies (No. AHI0501; Tago, Camarillo, CA) or medium (control) in 4 ml tubes (Greiner, Frickenhausen, FRG). Stimulations were carried out for 30 min at 37°C in a humidified atmosphere with 6% CO_2 . Reactions were

terminated by adding 300 μl ice-cold PBS per tube after which the samples were centrifuged (10 min, $500 \times g$, 4°C). Supernatants were decanted into separate tubes and pellets were resuspended in 500 μl PBS supplemented with 3.5% perchloric acid. All tubes were stored at -20°C before analysis of the histamine content by fluorometric autoanalysis. The percentage histamine release per tube was determined from the total histamine contained in pellet and supernatant. The net histamine release was calculated by subtracting the spontaneous release of cells incubated with medium alone.

2.4. IL-4 release

From the final basophil preparation, cells were transferred to sterile round-bottomed microtiter plates (Greiner) at 100,000 basophils per well and stimulated with various concentrations of anti-human IgE, as used for histamine release. After 4 h incubation at 37°C in a humidified atmosphere with 6% CO_2 , supernatants were harvested and stored at -20°C until IL-4 was assayed by a sandwich ELISA. Flat-bottomed microtiter plates (Maxisorp F96, Nunc, Wiesbaden, FRG) were coated overnight at 4°C with monoclonal mouse anti-human IL-4 (clone 8D4-8; PharmIngen, San Diego, CA) diluted 1/4000 in Tris (0.1 M Tris/HCl, 0.1 M NaCl, 2.5 mM MgCl_2 , pH 7.5). Between incubation steps, plates were washed $7 \times$ with Tris-T (Tris/0.05% Tween 20). Following incubation (90 min) with samples or serial dilutions of recombinant human IL-4 (No. 2181-01; Genzyme, Cambridge, MA) as a standard, biotinylated monoclonal rat anti-human IL-4 (clone MP4-25D2; PharmIngen) diluted 1/4000 in Tris-T/0.5% BSA was added for 60 min. Afterwards, the plates were incubated with streptavidin-alkaline phosphatase (1/1000 in Tris-T/0.5% BSA) for 60 min. The chromogen solution was 1 mg/ml *p*-nitrophenylphosphate in 0.1 M Tris/HCl, 0.1 M NaCl, 5 mM Mg/Cl_2 , pH 9.5. The absorption was read at 405 nm. The sensitivity of the IL-4 ELISA was 50 pg/ml and the upper detection limit 3.3 ng/ml.

2.5. Flow cytometry

Some 100,000 cells suspended in NaN_3 -PBS (15 mM NaN_3 in PBS) were stained on ice for

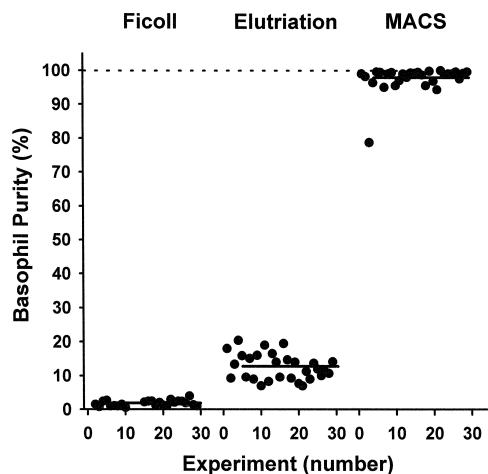


Fig. 1. Purity of basophil preparations after Ficoll density centrifugation, counterflow elutriation and negative selection by magnetic cell sorting (MACS). Each dot represents one donor ($n = 30$); bars depict mean values.

20 min with phycoerythrin (PE) conjugated mouse anti-CD123 (9F5) and fluorescein isothiocyanate (FITC) conjugated mouse anti-HLA-DR (L243, Becton Dickinson, Heidelberg, FRG). As isotype control, PE conjugated mouse IgG1 and FITC conjugated mouse IgG2a (Dako, Hamburg, FRG) were

employed at concentrations recommended by the manufacturer. After washing with 1 ml NaN_3 -PBS, the pellets were resuspended in 100 μl NaN_3 -PBS, and mixed with 100 μl NaN_3 -PBS/3% PFA to fix the cells. Following incubation for 10 min, the cells were centrifuged ($400 \times g$, 4°C , 10 min) and resuspended in 200 μl NaN_3 -PBS. Flow cytometric analysis of immunofluorescence-labelled cells was performed with a FACSCalibur (Becton Dickinson).

3. Results

3.1. Purity of basophil preparations

The mononuclear cell preparations (MNCs) obtained by the Ficoll gradient contained basophils with a mean purity of $1.8 \pm 0.83\%$ (Fig. 1) with most of the eosinophils and neutrophils removed. Following elutriation, the mean purity of the basophil-rich fractions increased to $12.6 \pm 3.87\%$ (Fig. 1). Contaminating cells were mainly monocytes, but lymphocytes, a few neutrophils and very few eosinophils were also detected as determined by microscopic inspection of May-Grünwald-stained

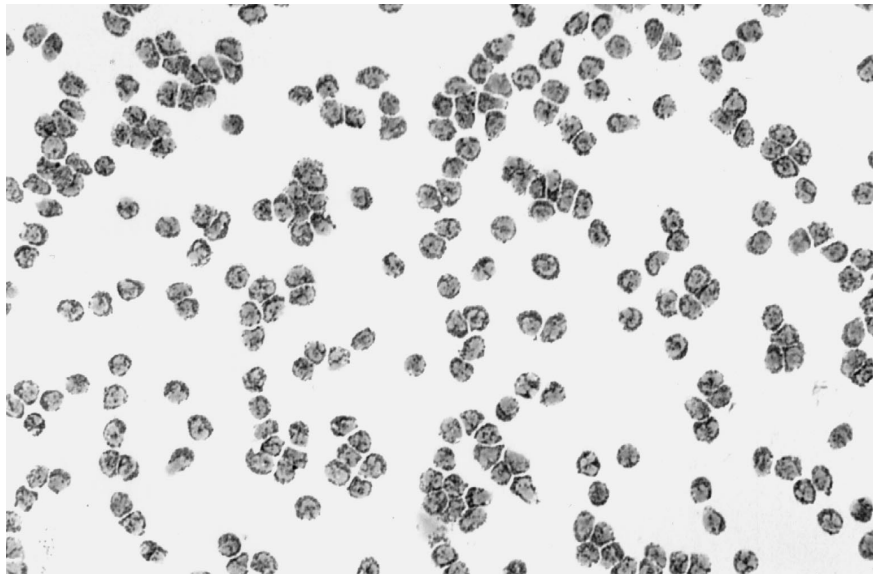


Fig. 2. Purified basophils stained with May-Grünwald (original magnification $\times 160$).

cytospins. After purification of basophils with the MACS system, the mean purity rose to $97.6 \pm 3.96\%$ (Fig. 1) with a range of 78.7–100%. The median was 99%. Light microscopical examination revealed normal morphology of the basophils (Fig. 2) without signs of degranulation (Fig. 3). In general, the few remaining contaminating cells had a monocytic appearance. The viability determined by Trypan blue staining was $99.6\% \pm 0.83\%$. The purity of basophils after MACS (measured by counting 1000 cells of May–Grünwald-stained cytospins) was confirmed by the analysis of 10,000 cells by flow cytometry in a random test of seven cases. Basophils express the IL-3 receptor CD123 (Agis et al., 1996; Baghestanian et al., 1996) but not HLA-DR (Bodger and Newton, 1987). All the other cell types expressing CD123 also express HLA-DR. Therefore, the basophils were detected as CD123 positive and HLA-DR negative cells. A typical example is shown (Fig. 4) where the purity determined by flow cytometry was 99.04% in comparison to 99.2% when assessed by staining with May–Grünwald.

3.2. Yield of basophil preparations

Since the low content of basophils in peripheral blood hampers accurate assessment of the quantity of basophils, the basophil recovery rate was calculated

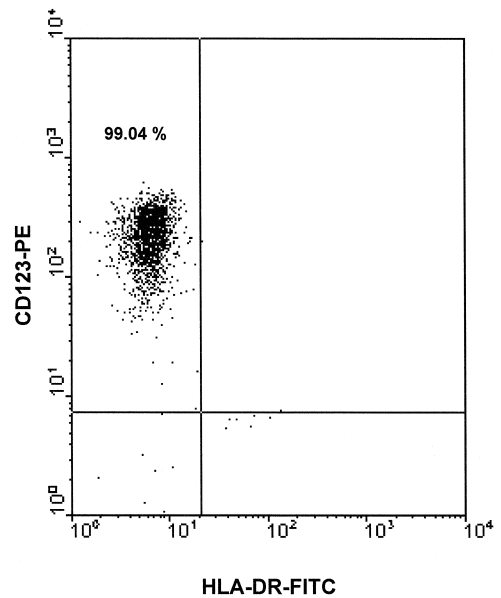


Fig. 4. Dot plot illustration of flow cytometry of purified basophils. The purity of 99.2% as determined by May–Grünwald stain was confirmed by flow cytometry. The cells were stained for HLA-DR (FITC) and CD123 (PE) and subsequently analysed on a FACSCalibur. Basophils express CD123 but not HLA-DR. Some 99.04% of the cells showed this pattern (one representative example out of seven is shown).

as the total number of basophils in MNCs as compared to the number of basophils after elutriation and

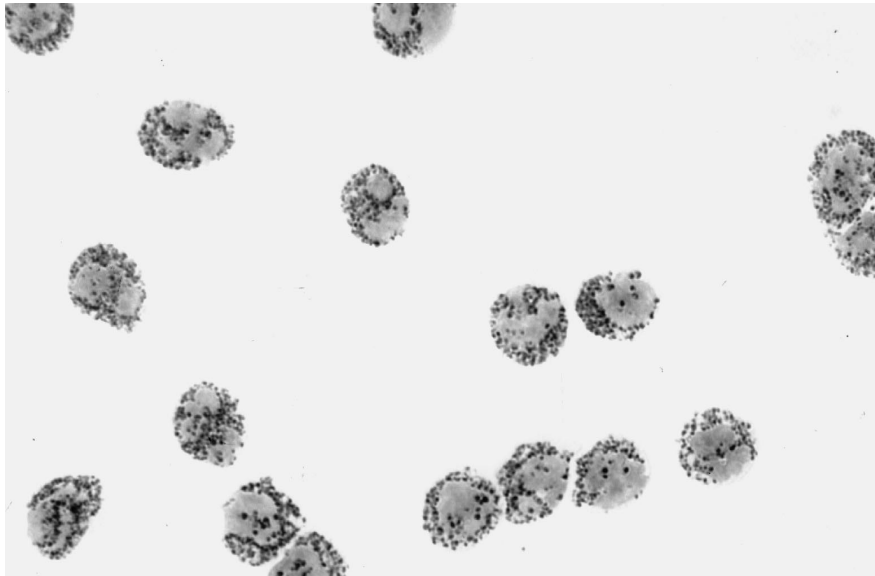


Fig. 3. Purified basophils stained with May–Grünwald (original magnification $\times 1008$).

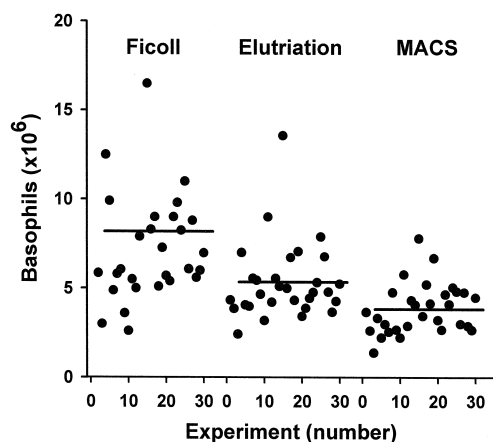


Fig. 5. Recovery of basophils after Ficoll density centrifugation, counterflow elutriation and negative selection by magnetic cell sorting (MACS). Each dot represents one donor ($n = 30$); bars depict mean values.

final selection with the MACS system. The recovery rate of basophils was $68.7 \pm 20.5\%$ after counterflow elutriation and $49.7 \pm 15.6\%$ after MACS (Fig. 5). The mean recoveries were 4.79×10^6 basophils and 3.44×10^6 basophils, respectively.

3.3. IgE-mediated histamine release

To investigate whether the purification procedure interfered with the functional capacity of the ba-

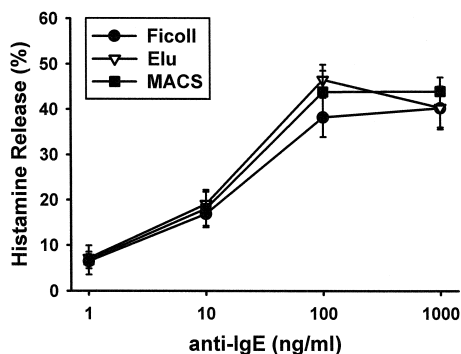


Fig. 6. Histamine release from human basophil preparations ($n = 7$) obtained after Ficoll, elutriation and MACS. Cells were stimulated with various concentrations of anti-human IgE and culture supernatants were recovered after 30 min for mediator determination. Spontaneous histamine release ($7.17 \pm 2.69\%$, $9.73 \pm 3.98\%$, and $8.11 \pm 1.87\%$, respectively) was subtracted from the corresponding results. Bars depict standard deviations.

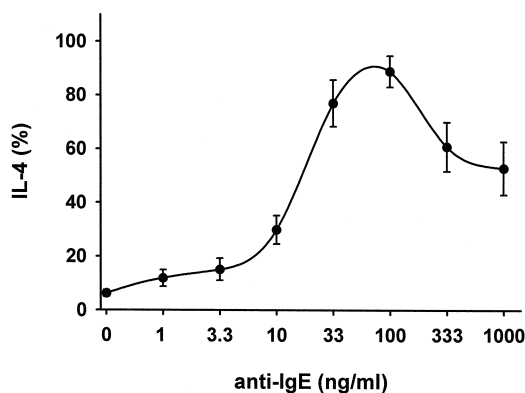


Fig. 7. Dose-dependent IL-4 production of purified human basophils ($n = 10$) after stimulation with anti-human IgE. Since absolute IL-4 concentrations varied between donors (160–1960 pg/ml), the results are depicted in percentage of the maximal IL-4 response. Bars represent standard errors.

sophils, the anti-human IgE induced histamine release from cells after the individual purification steps was compared. As shown in Fig. 6, no significant dose–response differences were found between the three stages of purification. The mean spontaneous histamine release was less than 10% and the maximal mean response was 46.55% at the optimal concentration of anti-IgE.

3.4. IgE-mediated IL-4 production

To determine whether the capacity of basophils to produce IL-4 would be affected by the purification procedure, the final preparations were stimulated with various concentrations of anti-human IgE (Fig. 7). Supernatants were recovered after 4 h and assayed for IL-4 production. The dose-dependent IL-4 production was maximal at 100 ng/ml IgE (range 160–1960 pg/ml).

4. Discussion

In this report, we present an easily reproducible and rapid method to purify basophils close to homogeneity, within 3.5 h. This high degree of purity may

be required for studying signal transduction and metabolic or immunological processes exclusively for basophils, and when sensitive molecular genetic techniques such as PCR are utilised. Furthermore, the determination of parameters characteristic not only of basophils, such as cytokine production or expression of certain surface markers, is facilitated by the high purity of basophils obtained with this method. Purified basophils were intact both morphologically (showing no degranulation) and also functionally as assessed by anti-IgE-induced histamine release, a process which was unaffected by the purification procedure. In addition, IL-4 production by purified basophils activated with various stimuli such as anti-IgE, ionomycin and soluble antigen from *Schistosoma mansoni* eggs (data not shown) gave rise to results comparable to those obtained with our previous methods (Falcone et al., 1996; Gibbs et al., 1996).

Since it can be performed at a lower cost, the technique described in this paper is a clear improvement over our previously published protocols (Falcone et al., 1996; Gibbs et al., 1997). The crucial improvement is the replacement of the immunomagnetic beads by the MACS system that increased the mean purity to 97.6% and mean recovery rate to nearly 50% with a viability of 99.6%. The mean absolute number of purified basophils obtained from 216 ml blood was 3.44×10^6 . We also purified basophils from smaller blood samples (70 ml) with comparable purities and slightly higher losses (data not shown). When we purified basophils from buffy coats, we obtained similar results as with purification of basophils from freshly drawn blood. The MACS procedure was essentially performed as recommended by the manufacturer, although, we did not add EDTA to the PBS-BSA buffer since the presence of EDTA may lead to a significant reduction of ionomycin-induced IL-4 production from basophils (data not shown).

Several basophil purification techniques have been published over the last decades. The majority of these involve an enrichment step using Ficoll or Percoll density centrifugation followed by either positive or negative selection. However, there are a number of problems associated with these techniques in terms of achieving both high purities together with high yields and functionally intact basophils. The

most direct method is positive basophil selection via $\text{Fc}\epsilon\text{RI}$ or CDw17. However, although this may give rise to purities of over 95%, several authors have shown that positive selection causes a substantial reduction of histamine release from the cells (Weil et al., 1983; Graziano, 1988; Schroeder and Hanrahan, 1990; Willheim et al., 1995). A further problem of selection through the IgE-receptor is the co-purification of B lymphocytes carrying membrane IgE and of activated macrophages and eosinophils which both express the high affinity IgE receptor.

Basophil purification by negative selection is not usually affected by the problems of direct cell activation, but has been crucially dependent on successful prior basophil enrichment. Given their relatively small numbers in whole blood, this enrichment is vital in terms of eliminating the bulk of erythrocyte and granulocyte contaminants. This is achieved either by dextran sedimentation or density centrifugation using Percoll, Ficoll or a mixture of both. Of these, Ficoll density centrifugation is the swiftest and least cumbersome method giving rise to relatively erythrocyte-free basophils with purities ranging from 1 to 5%. These purities may be sufficient to warrant direct further purification using the MACS system although lower final purities are obtained (range 66–95%) compared to the procedure described in this paper. Moreover, the costs are considerably greater due to the much higher amounts of MACS reagents required. When density centrifugation is followed by counterflow elutriation, virtually all remaining erythrocytes and thrombocytes as well as many mononuclear cells are removed, resulting in basophil purities of over 10%. In our hands, an enrichment of the basophils to more than 10% prior to using the MACS system is essential to obtain reproducible purities exceeding 99%. Instead of Ficoll followed by elutriation, other enrichment techniques may be used before the MACS system that may give rise to similar purities as those obtained with our method, but it still has to be established whether these techniques result in the same yields. Many reports have employed Percoll gradients where purities exceeding 30% may be achieved. However, the use of differential Percoll gradients is cumbersome. In agreement with our own observations (Gibbs et al., 1997), Bjerke et al. (1993) have reported that the success of Percoll gradient enrichment is largely

limited to the use of freshly drawn blood and homogeneous basophil density. Furthermore, in contrast to Ficoll density centrifugation, where over 70% of basophils are recovered, a large proportion of basophils are lost using discontinuous Percoll gradients.

Following prior enrichment, many reports have used flow cytometry to obtain highly purified basophils. However, though leading to high purities, yields are low and these techniques are more suitable for small blood samples since they are costly and time consuming. We and others have previously employed negative selection using Dynabeads directed against CD2-, CD14-, CD16- and CD19-positive cells (Mul et al., 1992; Falcone et al., 1996; Gibbs et al., 1996, 1997). However, the success of this technique was more dependent on prior basophil enrichment than the MACS system and though applicable to the purification of large numbers of basophils from a variety of donors, it gave rise to lower levels of purity. The higher purities obtained with the MACS system could be due to the small size of the superparamagnetic beads (about 50 nm in diameter) leading to a much bigger surface area for interactions between antibodies and cells, and the strong magnetic fields generated by the magnet and the column material. This leads to retention of weakly labelled contaminating cells expressing low numbers of surface markers (Miltenyi et al., 1990). Furthermore, the MACS system, which contains antibodies against eight surface markers, is currently more economical than purchasing each antibody separately.

In conclusion, we have developed a method to purify human basophils that is simple, rapid and results in a nearly pure functionally intact population of basophils with good yields. It is applicable to large blood samples and is economical. This method should facilitate future investigations on the biological and pathophysiological role of basophils in allergy and inflammation.

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