

Seedlings
Salvaged

CHAPTER II

AN IMPROVED METHOD FOR THE PURIFICATION OF BASOPHILIC GRANULOCYTES FROM HUMAN BLOOD

Frederik P.J. Mul, Edward F. Knol and Dirk Roos

32079

Studies on human basophils are hampered by the low number of basophils in peripheral blood. Here we describe the purification of human basophilic granulocytes with immunomagnetic beads to improve an already established method of purification. A 70% pure basophil suspension was incubated with monoclonal antibodies (CD2, CD14, CD16 and CD19) recognizing the contaminating cells. After incubation with magnetic susceptible beads coated with goat-anti-mouse-IgG, the bead-cell rosettes were removed by a magnet. In this way, the basophil purity increased to 94%. The loss of basophils during the bead procedure was about 20%. The amount of histamine per basophil and the spontaneous release of histamine during subsequent incubation of the cells was not altered by the purification procedure. The kinetics and the dose response of histamine release after addition of anti-IgE or FMLP was also unchanged. Binding of CD63 was not altered, indicating that the purification procedure did not result in activation of the basophils.

This improved method for purification of human basophils now permits measurement of non-basophil-specific parameters, such as changes in intracellular free Ca^{2+} , without the problem of interference by contaminating cells.

1. INTRODUCTION

Basophilic granulocytes (basophils) are known to participate in a number of allergic and inflammatory reactions by releasing mediators such as histamine, leukotrienes and chemotactic factors, in response to various stimuli (Peters et al., 1987). Although less than 1% of all circulating leukocytes are basophils, we routinely obtain 70% pure basophils in about 60% yield by successive isopycnic centrifugation and counter-flow elutriation (de Boer et al., 1986). However, the remaining contaminating leukocytes may interfere with the histamine release from the basophils, as shown for example by the effect of platelet activating factor (Colombo, 1990). Also, when non-basophil-specific parameters are analyzed, such as changes in intracellular free Ca^{2+} , contaminating cells may influence the signals measured.

Further purification of the basophils by physical methods results in a low recovery and a selection within the basophils. However, new isolation methods have recently been published based on a negative selection procedure through incubation of cell suspensions with monoclonal antibodies recognizing the contaminating cells. After subsequent incubation with magnetic susceptible beads coated with goat-anti-mouse-IgG, the bead-cell rosettes can be separated rapidly and efficiently with a magnet (Funderund, 1987). This technique can be used for isolating large numbers of cells at a relatively high yield, and without selecting a subpopulation of the desired cells (Bach et al, 1990). In the present study we describe the adaptation of this method for purification of human basophils.

2. MATERIALS AND METHODS

2.1 Materials.

Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden), human albumin (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB), Amsterdam, The Netherlands), sheep serum containing IgG antibodies against human IgE (CLB, no SH25P01), monoclonal antibodies against IgE (no I-6510, Sigma Chemicals, St.Louis, MO, USA), CD2 (CLB-T11), CD14 (CLB-mon/1), CD16 (CLB-gran/11) and CD19 (CLB-B4) were obtained from the manufacturers without the presence of a preservative. The concentration of anti-IgE antibodies in the sheep serum was calculated by comparing affinity-purified sheep anti-human IgE antibodies with serum dilutions for histamine release from human basophils. Goat-anti-mouse-IgG-coated Dynabeads were obtained from Dynal A.S.(Oslo, Norway). HEPES was obtained from Sigma Chemicals. Bovine serum albumin (BSA) was purchased from Organon Teknica B.V. (Oss, The Netherlands). Formyl-methionyl-leucyl-phenylalanine (FMLP) (Sigma Chemicals) was dissolved in DMSO and stored at -20°C . FMLP was diluted at least 300-fold in the cell incubations. The final concentration of DMSO ($\leq 0.3\%$, v/v) had no effect on cell viability or histamine release.

2.2 Purification of basophils by centrifugation

The basophils were purified as previously described by De Boer and Roos (1986) with minor modifications. In short, one unit (500 ml) of human blood from a healthy donor without an allergic history was collected in a plastic triple-bag with 75 ml of ACD (100 mM anhydrous

disodium citrate and 128 mM anhydrous glucose; pH 5). The blood was centrifuged (5 min, 4000 X g, 20°C) and the plasma was separated from the cells. The upper 50 to 100 ml of the packed cells (the "buffy coat"), were collected and diluted to about 240 ml with phosphate-buffered saline (PBS) containing 13 mM trisodium citrate. This cell suspension was distributed over eight 50-ml centrifuge tubes. Subsequently 12 ml of isotonic Percoll with a specific gravity of 1.077 g/cm³ was carefully injected underneath the cell suspension in each tube. After centrifugation (20 min, 1000 X g, 20°C) the interfaces were collected, combined and injected in an elutriator centrifugation system (Beckman J21C centrifuge with a JE-6 elutriation rotor). The elutriation medium consisted of PBS with 13 mM trisodium citrate and 5 mg of human serum albumin per ml. During the elutriation procedure the third fraction was collected between 2550 and 1000 rpm. This fraction was further purified by centrifugation over a discontinuous Percoll gradient of 1.068 g/cm³ and 1.075 g/cm³ (18 min, 1000 X g) at room temperature. The purity of the basophils in this preparation was about 70%. The number of cells in this preparation was about 15*10⁶. The cells were counted electronically (Coulter counter model ZF; Coulter Electronics, Dunstable, UK). The basophil content was determined by inspection of the cells after differential staining with Alcian blue (Gilbert et al., 1975) and by inspection of cytocentrifuge preparations stained with May-Grünwald/Giemsa.

Platelets were not present, as determined with MoAb Y2 against platelets GP IIIa (Dakopatts, Copenhagen, Denmark) and by electron microscopy.

2.3 Purification of basophils with Dynabeads.

Basophils (70% pure) were incubated for 30 minutes at 4°C in 3 ml of incubation medium (132 mM NaCl, 6 mM KCl, 1 mM MgSO₄, 1.2 mM potassium phosphate, 20 mM HEPES, 5.5 mM glucose and 0.5% (w/v) human albumin, pH 7.4) with a mixture of monoclonal antibodies against the contaminating cells (CD2, CD14 and CD16 at 1 µg/ml and CD19 at 2 µg/ml). After this incubation the cells were washed in incubation medium, resuspended in 3ml incubation medium and incubated in a head-over-head rotator (angle 10°) for one hour at 4°C with goat-anti-mouse-IgG-coated Dynabeads (ratio beads: cells=5:1). These Dynabeads (Dyna: prod. No. 11005) are magnetic susceptible beads. The contaminating cells, adhering to the beads are attracted and kept in place on the wall of the test tube by the magnetic field of a magnet also obtained from Dynal (prod. No. 12001). After depletion of the immunomagnetic rosetted cells (this takes about 1-2 minutes), the cells left in the supernatant were collected. The beads were resuspended very gently with incubation medium to liberate enclosed basophils, and the mixture was placed again on the magnet to separate the beads from the remaining basophils. After addition of this supernatant to the first supernatant, the cell suspension was washed and resuspended in incubation medium supplemented with 1 mM CaCl₂. The purity of this final cell suspension was determined by staining with Alcian blue and with May-Grünwald/Giemsa as described above.

2.4 Histamine release.

Purified basophils were washed with and resuspended in incubation medium (0.3*10⁶/ml). Incubations were performed in 300 µl of incubation medium supplemented with 1 mM CaCl₂.

Before reagents were added, the cells were prewarmed for 10 minutes at 37°C. After addition of the stimulating agents, the cells were centrifuged at the indicated times for 10 sec at 12000

x g, and 250 μ l of supernatant was collected. These supernatants were mixed with 200 μ l of perchloric acid (12 N) and 750 μ l of saline, and stored at 4°C. For measurement of the total amount of histamine, 10^5 cells were lysed in perchloric acid. Histamine was measured by fluorometric analysis as described by Siraganian (1975). Histamine release was calculated as percentage of the total amount of histamine in the cells.

2.5 Immunofluorescence.

Cells were washed and resuspended in PBS with 1% (v/v) BSA to a concentration of 10^7 per ml. Twenty μ l of this suspension was incubated with 20 μ l of the monoclonal antibodies in the appropriate dilutions. Incubations were performed at 4°C during 30 min. The cells were then washed once with PBS supplemented with 1% (v/v) BSA, and fluoresceinated antibody against mouse immunoglobulin was added, followed by another incubation for 30 minutes at 4°C. Subsequently, the cells were washed again with PBS, 1% BSA, and analyzed by flowcytometry in a FACScan flowcytometer (Becton and Dickinson, San José, CA, USA). The percentage of positive cells was expressed as the percentage of cells with a fluorescence intensity above the maximal background of a control IgG1 antibody against trinitrophenyl. This threshold is indicated by a vertical bar in the FACScan pictures.

3. RESULTS

After elutriation, the basophil suspensions were still contaminated with lymphocytes, monocytes, neutrophils and blast-like cells, as determined by microscopic inspection of cytopspin preparations stained with May-Grünwald/Giemsa. The purity of the basophils was 50-80%, as detected by flowcytometric analysis with a MoAb against IgE (fig 1A) and by microscopic inspection of cells stained with Alcian blue (Table I). About 20% of the cells bound CD2, directed against the T11 antigen on T cells and most NK cells (fig 1B). About 13% of the cells bound CD14, directed against the receptor for LPS-binding protein which is present on monocytes and macrophages and (weakly) on neutrophils (fig 1C). About 12% of the cells bound CD16, directed against the low-affinity receptor for IgG (Fc γ RIII) on neutrophils, macrophages and NK cells (fig 1D). About 3% of the cells bound CD19 directed against a pan-B cell antigen (fig 1E). With these 5 MoAbs about 98% of the cells were characterized.

The cells (5×10^6) were incubated with a mixture of CD2, CD14, CD16 and CD19 for 30 minutes at 4°C. Ca^{2+} was omitted to reduce spontaneous activation of the basophils. To determine the optimal concentration of the MoAbs, several concentrations were tested on different mixtures of lymphocytes, monocytes, neutrophils and basophils. Concentrations were chosen that caused optimal removal of the first three cell types in the subsequent Dynabead procedure and minimal loss of basophils (data not shown).

After incubation with magnetic susceptible beads coated with goat-anti-mouse-IgG, the bead-cell rosettes were separated rapidly and efficiently with a magnet. The final preparations contained about 94% basophils (Table I). The remaining contaminating cells were mainly eosinophils and blast-like cells (characterized as metamyelocytes) that were not recognized by

with 200 μ l of
 ent of the total
 measured by
 calculated as

of 10^7 per ml.
 ibodies in the
 ells were then
 tibody against
 inutes at 4°C.
 flowcytometry
 percentage of
 isity above the
 old is indicated

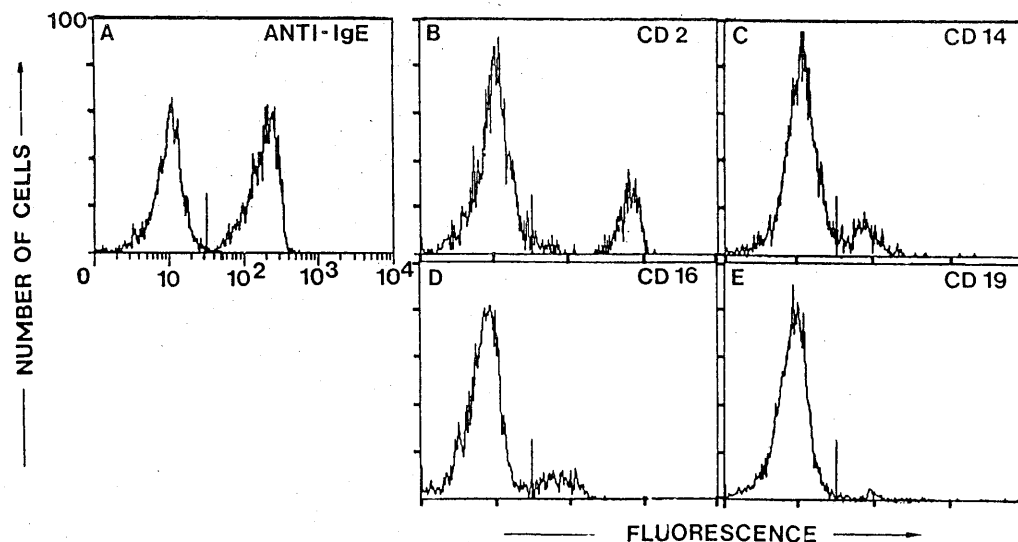


Figure 1: Characterization of the cell suspension before the Dynabead procedure with specific MoAbs. The cells were incubated with MoAb anti-IgE (1:500) (fig 1A), CD2 (1mg/ml) (fig 1B), CD14 (1 mg/ml)(fig 1C), CD16 (1 mg/ml)(fig 1D) or CD19 (2 mg/ml)(fig 1E). Subsequently, FITC-labeled goat antibody against mouse Ig was added. Binding was analyzed by flow cytofluorometry. Fluorescence intensity is in arbitrary units. The vertical bar indicates the maximal background fluorescence with an irrelevant antibody (IgG1 antibody against TNP). The positive cells are those with fluorescence intensity above the background.

is, monocytes,
 of cytopsin
 as 50-80%, as
 y microscopic
 CD2, directed
 ie cells bound
 monocytes and
 CD16, directed
 d NK cells (fig
 E). With these

I CD19 for 30
 basophils. To
 ere tested on
 ntrations were
 ent Dynabead

IgG, the bead-
 l preparations
 were mainly
 recognized by

the selected mixture of MoAbs and were also enriched by this method. Removal of these contaminating cells was not possible due to the lack of specific MoAbs against these cells. Experiments with CD67 MoAbs, reactive with eosinophils, showed a dramatic decrease in the yield of basophils, probably because these MoAbs bound to some extent also to basophils.

The loss of basophils during the Dynabead procedure was about 20% (Table I), probably caused by the washing steps. Analysis of the basophils by immunofluorescence showed no reaction with the MoAbs used for purification. More than 95% of the cells bound the MoAb against IgE (Fig 2). From these results we conclude that the chosen concentrations of the different MoAbs caused only a slight loss of basophils by non-specific binding and were high enough to remove 80% of the contaminating cells.

The amount of histamine in the basophils and the amount released spontaneously after incubation of the cells for 45 min at 37°C showed that the whole purification procedure caused no loss of histamine (Table I) (De Boer et al., 1986).

To exclude the possibility that the procedure interferes with the functional capacity of the basophils we compared the histamine release induced by anti-IgE (Lichtenstein et al., 1971) or FMLP (Siraganian et al, 1977) before and after the bead purification. As shown in figures 3A and 3B, no significant dose response differences were found. The kinetics of the FMLP- and anti-IgE-induced histamine release before and after the bead purification were also identical (fig. 4A and 4B).

TABLE I

BASOPHIL PURIFICATION FROM THE BLOOD OF TEN DIFFERENT DONORS

Donor (nr)	Before	After Dynabead procedure			
	Basophil purity (%)	Basophil purity (%)	Basophil yield (%)	Histamine per basophil (pg)	Histamine spontaneous release (%)
1	80	92	78.3	1.32	2.5
2	78	95	75.8	1.25	3.8
3	80	98	85.0	1.48	2.3
4	83	96	84.6	1.38	2.4
5	52	98	85.0	1.79	3.4
6	84	95	78.4	1.55	5.7
7	60	93	72.0	1.81	2.5
8	55	90	N.D.	1.52	3.3
9	68	87	83.0	1.43	4.4
10	75	98	80.5	1.41	4.7
Mean	71.5	94.2	80.2	1.5	3.50
± SD	11.4	3.5	4.3	0.17	1.09

Basophil purity was determined by microscopic inspection of cells stained with Alcian blue. Histamine content per basophil before Dynabead procedure was 1.6 ± 0.3 pg/cell (mean $\pm$ SD, $n=22$). N.D.; Not determined.

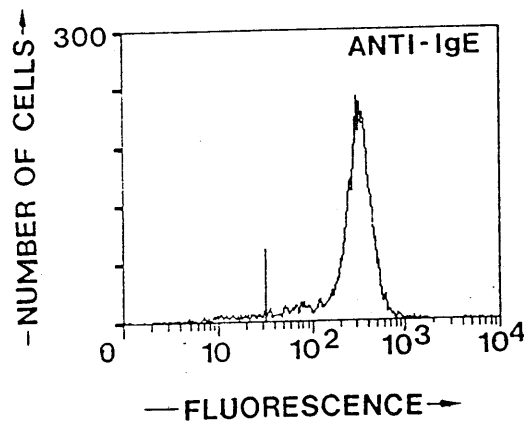


Figure 2: Binding of MoAb anti-IgE to the cell suspension after the Dynabead procedure. The cells were incubated with MoAb anti-IgE (1:500) and FITC-labeled goat antibody against mouse Ig. Binding was analyzed by flow cytometry. Fluorescence intensity is in arbitrary units. The vertical bar indicates the maximal background fluorescence with an irrelevant antibody (IgG1 against TNP). The positive cells are those with fluorescence intensity above the background.

3 DONORS

3 S
(%)

100 µg/ml cyan blue.
1 (mean ± SD,

Ab anti-IgE to
the Dynabead
are incubated
30) and FITC-
against mouse
zed by flow
:ense intensity
e vertical bar
background
in irrelevant
t TNP). The
those with
above the

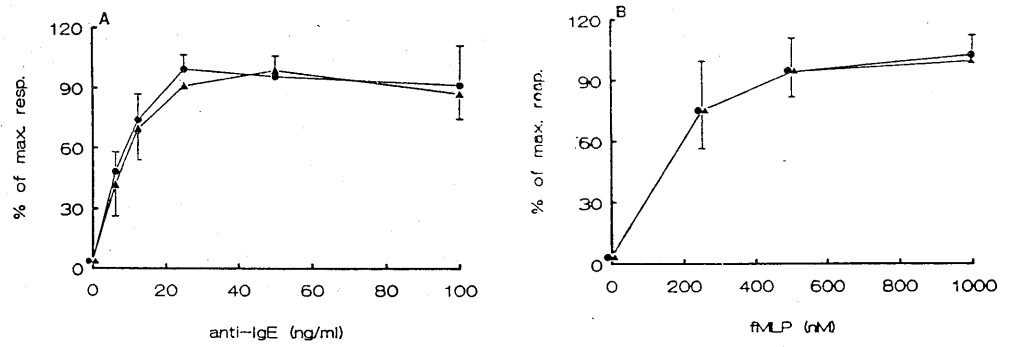


Figure 3: Histamine release induced by a concentration range of anti-IgE (3A) or FMLP (3B). Results are expressed as the mean $\pm$ s.d. of three experiments performed with cells from 3 different donors. ($\blacktriangle$), before the bead procedure; ($\bullet$), after the bead procedure. Concentrations of the stimulating agents were lowered by 1:1 dilutions in PBS in five steps, starting at 100 ng anti-IgE/ml or in three steps starting at 1 μ M FMLP. Basophils were incubated for 45 min at 37 $^{\circ}$ C with the stimulating agents, and the histamine release was determined in the cell-free supernatant. Histamine release was calculated as percentage of the response of the suspension before the bead procedure. Maximal response in the three experiments was 21.3%, 41.4% and 31.4% of the total histamine content for anti-IgE and 66.0%, 72.9% and 69.9% for FMLP.

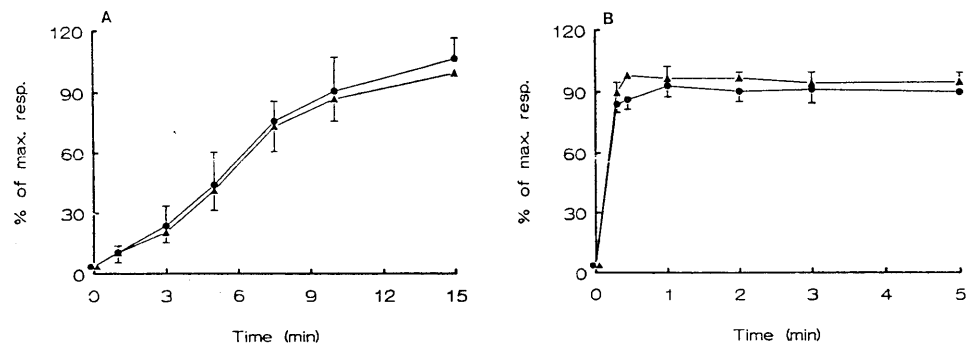


Figure 4: Time course of anti-IgE-induced (4A) or FMLP-induced (4B) histamine release. Results are presented as the mean $\pm$ s.d. of three experiments performed with cells from 3 different donors. The cell suspensions were stimulated with 100 ng of anti-IgE per ml or 1 μ M FMLP, before ($\blacktriangle$) or after the bead procedure ($\bullet$). Histamine release was determined in the cell-free supernatant of basophils after the indicated intervals and calculated as percentage of the maximal histamine release of the suspension before the bead procedure. Maximal response in the three experiments was 24.5%, 52.1% and 22.5% of the total histamine content for anti-IgE and 81.0%, 82.9% and 62.7% for FMLP.

Previously, we have shown that binding of a monoclonal antibody against the CD63 epitope increased 20-100 fold after activation with fMLP or anti-IgE (Knol et al, 1991). Figure 5 shows that binding of this MoAb did not change due to the bead purification. The small shift to the right was probably caused by a slight increase in the background binding of the control MoAb (not shown). Figure 6 shows the binding of CD63 after bead purification and stimulation with anti-IgE (100 ng/ml). The cells reacted with a 30-40 time increase in CD63 binding (87.0% histamine release).

Figure 5: Binding of CD63 antibodies to basophils before and after the bead purification. The basophils were incubated with CD63 antibodies and FITC-labeled goat antibody against mouse Ig. (—), before the bead procedure; (.....), after the bead procedure. Fluorescence intensity is in arbitrary units. Ninety-ninepercent of the bead-purified basophils showed a background fluorescence with an IgG1 antibody against TNP below the maximal binding of this antibody to non-bead-purified basophils (indicated by the vertical bar).

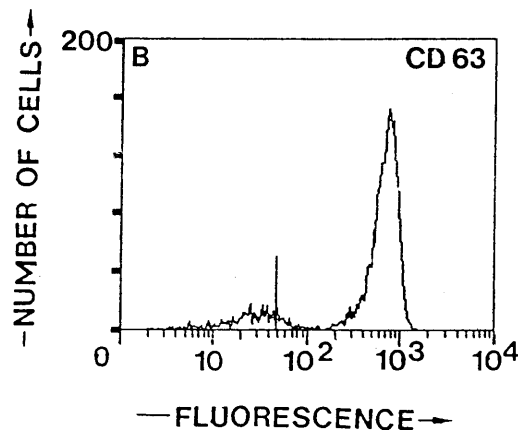
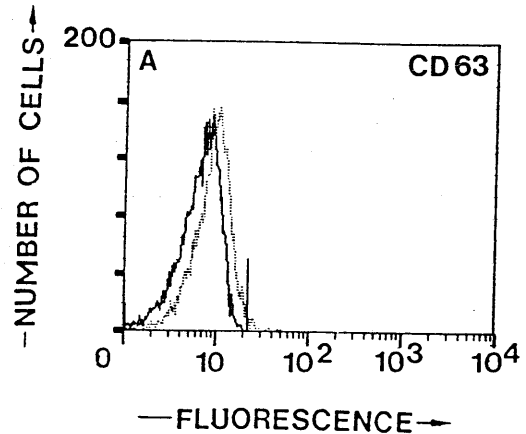


Figure 6: Binding of CD63 antibodies to basophils after bead purification and stimulation with 100 ng of anti-IgE per ml. The basophils were incubated with MoAb CD63 and FITC-labeled goat antibody against mouse Ig. Fluorescence intensity is in arbitrary units. The positive cells are those with fluorescence intensity above the maximal background fluorescence with IgG1 anti-TNP indicated by the vertical bar.

We also tried to use this bead purification on mononuclear leukocytes obtained from small amounts of blood (30 ml). This resulted in 41.9 + 16% (n=5) pure basophils with a low recovery. This was probably caused by large cell-aggregates formed by the bead-cell rosettes (not shown).

4. Discussion

Studies on basophil functions are hampered by the interference with contaminating cells. The purity of human basophils after isopycnic centrifugation and elutriation varies between 50 and 80%. Previously, we found that these cells cannot be used for studies on non-basophil-specific parameters, due to the interference of the contaminating cells. Therefore, we developed a method that yields an almost pure population of basophils. This method circumvents the donor-dependent specific gravity of the cells, and therefore leads to a high purity (>90%). The recovery of the bead procedure is about 80%; the overall recovery from the buffy coat is about 50%. The inertness of this method is also remarkable. This was tested by comparing the purified and the contaminated basophil suspensions for histamine content and spontaneous and stimulus-induced histamine release. These parameters showed no significant differences between the two suspensions. Comparison of the expression of an activation marker (CD63) on the basophil membrane between these two suspensions also showed no significant difference.

Our method differs from methods that use positive selection, e.g. with monoclonal mouse IgE to trinitrophenol (TNP), as published by Schroeder et al. (1990). Although this method leads to a high basophil purity, functional responses of the basophils are dramatically inhibited, as shown by a decrease of more than 50% in the anti-IgE-induced histamine release. This is probably caused by the presence of the mouse monoclonal IgE, which may inhibit the crosslinking of human IgE on the membrane of these basophils. Affinity chromatography, used by MacGlashan and Lichtenstein (1980) has limited applicability, because of the supply of human penicillin-specific IgE and the donor dependency of the anti-BPO binding. Moreover, both methods carry the hazard of basophil activation during or after the purification. Other methods, such as fluorescence-activated cell sorting, differential adherence or centrifugation techniques with different Percoll gradients, are only useful for small cell numbers, have a low recovery, are not inert or result in a low purity (Kauffman et al., 1983; Degenhart, 1988).

Our method is an adaptation of related methods recently published by Pilling et al. (1989) and Bach et al. (1990), for purification of lymphocytes and eosinophils, respectively. These investigators concluded that the Dynabead method provides an attractive alternative to conventional methods of purification and is suitable to purify small or large amounts of cells. We adapted this method to improve our, already established, method for the purification of basophils from human blood. Unfortunately, it was not possible to purify basophils directly from mononuclear leukocytes with this method.

By the use of this improved method for purification of large amounts of human basophils it now becomes possible to measure non-basophil-specific parameters, without the problem of interference of contaminating cells. In addition, analysis of membrane markers on basophils that are also present on other leukocytes will also be facilitated by our method.

5. REFERENCES

- Bach, M.K., Brashler, J.R. and Sanders, M.E. (1990) Preparation of large numbers of highly purified normodense human eosinophils from leukapheresis. *J. Immunol. Methods* 130, 277-81.
- De Boer, M., Roos, D. (1986) Metabolic comparison between basophils and other leukocytes from human blood. *J. Immunol* 136, 3447-54.
- Colombo, M., Casolaro, V., Warner, J.A., MacGlashan, D.W.Jr, Kagey-Sobotka, A. and Lichtenstein, L.M. (1990) The mechanism of mediator release from human basophils induced by platelet-activating factor. *J. Immunol* 145, 3855-61.
- Degenhart, H.J., van der Maarel, J.W.M., Jongkind, H., Raatgeep, H.C. and Neijens, H.J. (1988) Properties of human basophils isolated by fluorescence-activated cell sorting, *J. Allergy Clin. Immunol.* 82, 455-61.
- Funderud, S., Nustad, K., Lea, T., Vartdal, F., Gaudernack, G., Stenstad, P. and Ugelstad, J. (1987) Fractionation of lymphocytes by immunomagnetic beads. In: G.G.B. Klaus (Ed.), *Lymphocytes. A Practical Approach*. IRL Press, Oxford, p. 55.
- Gilbert, H.S., Ornstein, L. (1975) Basophil counting with a new staining method using Alcian blue. *Blood* 46, 279-86.
- Kauffman, H.F., Levering, P.R., de Vries, K. (1983) A single centrifugation step method for the simultaneous separation of different leukocytes with special reference to basophilic leukocytes, *J. Immunol. Methods* 35, 1-7.
- Knol, E.F., Mul, F.P.J., Jansen, H., Calafat, J. and Roos, D. (1991) Monitoring of human basophil activation via CD63 monoclonal antibody 435, *J. Allergy Clin. Immunol.* in press.
- Lichtenstein, L.M. (1971) The immediate allergic response: in vitro separation of antigen activation, decay and histamine release. *J. Immunol.* 107, 1122-30.
- MacGlashan, D.W.Jr, and Lichtenstein, L.M. (1980) The purification of human basophils, *J. Immunol.* 124, 2519-21.
- Peters, S.P., Grant, J.A., Dvorak, A.M. and Lichtenstein, L.M. (1987) Mast cells and basophils. In: J. Bousquet, P. Godard, and F.B. Michel (Eds), *Highlights in Asthmology*. Springer Verlag, Munich. 44, 183.
- Pilling, D., Kitas, G.D., Salmon, M. and Bacon, P.A. (1989) The kinetics of interaction between lymphocytes and magnetic polymer particles, *J. Immunol. Methods* 122, 235-41.
- Schroeder, J.T. and Hanrahan, R.L.Jr. (1990) Purification of human basophils using mouse monoclonal IgE, *J. Immunol. Methods* 133, 269-77.
- Siraganian, R.P. (1975) Refinements in the automated fluorometric histamine analysis system. *J. Immunol. Methods* 7, 283-90.
- Siraganian, R.P., Hook, W.A. (1977) Mechanism of histamine release by formyl-methionine-containing peptides. *J. Immunol.* 119, 2078-83.