

A three-step procedure for the purification of human basophils from buffy coat blood

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Received 23 August 1996; returned for revision 31 October 1996; accepted by W. Lorenz 22 January 1997

Abstract. *Objective and Design:* We report a method for basophil purification from buffy coats, which avoids positive selection of the cells and gives rise to good purity, yield and functional integrity of the cells.

Subjects: Buffy coat blood (concentrated leukocyte fraction derived from 450 ml venipuncture donations) obtained from healthy blood donors ($n = 51$).

Methods: Basophils were enriched by a three-step process starting with Ficoll density centrifugation ($1.6 \pm 0.1\%$ basophil purity) followed by counter current centrifugal elutriation ($17.7 \pm 1.4\%$ basophil purity). The final stage involved negative selection using Dynal immunomagnetic beads directed against CD2, CD14, CD16 and CD19 positive cell contaminants. Functional integrity of which was assessed by comparing the anti-IgE or calcium ionophore A23187 induced histamine release from basophils obtained from each enrichment step. Furthermore, basophil morphology was investigated using light and electron microscopy.

Results: The final mean basophil purity of $67.3 \pm 1.4\%$ with a yield of $3.5 \pm 0.5 \times 10^6$ basophils and a recovery of $21.8 \pm 2.4\%$ was achieved. Net histamine release from basophils stimulated with optimal concentrations of anti-human IgE was $39.1 \pm 6.5\%$ after Ficoll centrifugation, $41.6 \pm 7.7\%$ following elutriation and $35.7 \pm 6.8\%$ from the final purified fraction. Additionally, basophils enriched with our method showed intact morphology by electron microscopy and were functionally active to non-immunological stimulation.

Conclusions: These results compare favourably with previous studies, which have often required the use of positive selection via the Fc ϵ RI receptor, which may result in cell degranulation, or cell sorting, which cannot be applied to large cell numbers. Our method provides a reproducible technique for basophil enrichment when large numbers of functionally intact basophils are required.

Key words: Basophil – Purification – Elutriation – Immunomagnetic bead – Histamine

Introduction

Basophils, like mast cells, have been implicated in allergic and inflammatory reactions by releasing a number of preformed mediators including histamine and de novo generated lipid mediators such as LTC₄ as well as cytokines such as interleukin-(IL)4 [1, 2]. Basophils are known to infiltrate tissues in allergic diseases such as atopic dermatitis and asthma and may so play a crucial role in the initiation and maintenance of these allergic diseases following Fc ϵ RI-crosslinking [3, 4]. However, since other leukocytes, such as macrophages and eosinophils, have also been shown to express Fc ϵ RI-receptors [5, 6] the enrichment of basophils is vital for the study of this particular cell type. This is of particular importance when, for example, studying cytokine generation and release as well as the effects of basophil priming and growth factors.

Basophil purification techniques have been reported by several different centres [2, 7–11]. Given the low proportion of these cells in peripheral blood and large donor variation there exists a need not only to optimize their purification but also to define the limits of the purification to a wide range of blood donors. Furthermore, it is imperative to maintain the normal basophil function without possible activation that may arise through positive selection of the cells or by factors produced from other cell contaminants. This is of particular importance since basophils are sensitive to priming by a number of cytokines, such as IL-3 and granulocyte-macrophage colony stimulating factor which have been shown to give rise to enhanced histamine release from the cells [12, 13].

Our aim was, therefore, to study the reproducibility of our purification method on a wide range of donors and to optimize the purity obtained whilst maintaining a good yield of functionally and morphologically viable basophils.

Materials and methods

Materials

The following were purchased: trypan blue (Flow Laboratories, UK); -anti-CD16 (DAKO, Denmark); Dynabeads (M-450-anti-CD2, -anti-CD14,

-anti-CD19), rat anti-mouse IgG1 (DynaL, Germany); RPMI (GIBCO BRL, UK); D-Glucose, HClO₄, KCl, NaCl, NaH₂PO₄ (Merck, Germany); OsO₄ (Paesel & Lorei, Germany); Ficoll-Paque, Percoll (Pharmacia Biotech, Sweden); Epon 812, glutaraldehyde, propylene oxide (Serva, Germany); alcian blue, anti-human IgE, BSA, calcium ionophore A23187, heparin, HEPES, May-Grünwald stain, o-phthalaldehyde, sodium cacodylate (Sigma Chemical Co., USA).

Methods

Basophil purification. Step 1: Ficoll density centrifugation. Freshly prepared buffy coat blood (concentrated leukocyte fraction derived from 450 ml venipuncture donations, which were depleted of erythrocytes and plasma by centrifugation in 63 ml citrate buffer) was collected within 1 h of blood donation. A small proportion of serum from each donor assessed for total serum IgE level (CAP-system, Pharmacia). The contents of the buffy coats (50 ml) were then diluted in Ca²⁺/Mg²⁺-free HEPES-buffered Tyrode's solution (HB) containing heparin (10 iU/ml) and layered over 4 × 50 ml tubes (Falcon) containing Ficoll (10 ml). All tubes were then centrifuged (460 × g, 20 min, 4°C) and the basophil-rich interface harvested. The basophil number and purity was observed by alcian blue staining, after which the cells were further purified by counter current centrifugal elutriation.

Step 2: Counter current centrifugal elutriation. Cell suspensions in Ca²⁺/Mg²⁺-free HB containing BSA (2 mg/ml) were loaded into the elutriation chamber (JE-6B rotor, Beckman Instruments inc., Palo Alto, USA) at a constant rate of 20 ml/min and at a rotor speed of 2450 rpm. After collecting 200 ml the rotor speed was then reduced by 50 rpm and a further 200 ml collected. This process was repeated down to 2300 rpm and all collected fractions discarded. The speed was then further reduced successively by stages of 20 rpm and by 100 ml intervals to 2260 rpm where the basophil-rich fraction remaining in the elutriation chamber was harvested.

Step 3: Immunomagnetic beads. These basophil preparations were then highly enriched by negative selection using Dynal beads against CD2, CD14 and CD19 positive contaminating cell types. Additionally, Dynal beads coated with antibodies for CD16 according to the method described by Bjerke et al. [9] were also employed in the negative selection step. The immunomagnetic beads, together with the cells, were incubated (1 h, 4°C) with rotation at a bead: cell ratio of 10:1. After this incubation, the beads binding to cell contaminants were removed from the, unbound, basophil-rich fractions by a magnetic particle concentrator (MPC-1, Dynal), the bead fraction resuspended in HB and the procedure repeated 4 times.

Histamine release experiments

Aliquots of purified, semi-enriched (elutriated) or unpurified (Ficoll-fraction) basophils (2 × 10⁵ basophils per tube) were suspended in HB (450 µl per tube) and incubated for 10 mins before being challenged with anti-IgE (1/10⁴–1/10⁶ dilution; 50 µl) or the calcium ionophore A23187 (10⁻⁶–10⁻⁸ M) for a further 30 min. At the end of the incubation period, the reactions were terminated by the addition of ice-cold HB (500 µl), all tubes centrifuged (200 g, 2 min), supernatants decanted off and HB was added (1 ml) to the cell pellets. After the addition of perchloric acid (70%, 50 µl) to all tubes, the tubes were then assayed for histamine content using a fluorimetric autoanalyser (Bran and Luebbe) and the percentage histamine release for each tube determined from the total histamine content in the sum of pellet and supernatant tubes. The net histamine release due to each stimulus was then calculated by subtracting the spontaneous release.

Electron microscopy

Basophils were prepared for electron microscopy in a modified protocol as reported by Dvorak et al. [14]. Briefly, suspensions of basophils were

fixed in Karnofsky's fixative (1 h, room temperature) after which the cells were washed in sodium cacodylate buffer (0.1 M, pH 7.4) and kept overnight at 4°C. The following day, the cells were centrifuged (300 g, 5 min) after which the pellets were resuspended warm, 4% low-melting agar (200 µl) and following further centrifugation (300 g, pulse) the agar, containing the pelleted cells, was placed on ice to set. Small sections (2 mm) of the cells set in agar were then incubated in sodium cacodylate buffer (0.1 M, pH 7.4) containing osmium tetroxide (2%) for 1 h at 4°C after which the cells were washed twice in cacodylate buffer. The cells were then dehydrated in a graded series of alcohols, incubated in propylene oxide (3 × 20 min) followed by 1:1 propylene oxide-Epon mixture (2.5 h) and finally embedded in Epon overnight. The next day, the cells were polymerised in an incubator (60°C, 24 h) after which sections were placed on copper grids and stained with lead citrate for electron microscopy (EM 910, Carl Zeiss, Germany).

Unless expressed otherwise, all values are shown as the mean ± SEM. Statistical significance was determined using a paired Student's t-test.

Results

Basophil purification

Step 1: Ficoll density centrifugation. This procedure enriched the basophils to 1.6 ± 0.1% with a yield of 12.0 ± 0.7 × 10⁶ basophils (n = 81). A relationship was observed when comparing the basophil number and purity following the first enrichment step with Ficoll as shown in Figure 1. This demonstrates the large donor variation and may be used as a guide to the likely success of further purification steps since the number or purity of basophils after Ficoll also correlated with the final purity and yield (results not shown). Although also dependent on experimental handling in each case, our initial observations indicated that buffy coats yielding less than 10 × 10⁶ basophils following the Ficoll step were less likely to give rise to satisfactory purity and number making further enrichment not worthwhile.

The use of Ficoll at this stage was employed in order to remove erythrocytes and a large proportion of eosinophils and neutrophils. Neither elutriation nor the use of Percoll gradient density centrifugation were found to be a good substitute for the Ficoll step for different reasons. Firstly, the total number of cells in whole blood is too large for the capacity of the elutriation chambers generally employed and, secondly, the use of Percoll gradients at this stage was found to be both time consuming and not very reproducible in terms of the purity obtained (own unpublished observations).

Step 2: Counter current centrifugal elutriation. A mean basophil purity of 17.7 ± 1.4% and number of 5.7 ± 0.6 × 10⁶ basophils was obtained at this stage (n = 51). Our elutriation protocol was initially adapted from a previously reported method [15] where the basophils were gradually eluted from the chamber by reducing the rotor speed. We observed a marked spread of basophil fractions eluted over a wide range of rotor speeds and the procedure for counting all these fractions time consuming. In most cases, in order to maintain a high yield, it was necessary to pool various basophil fractions thus compromising the higher purity. We therefore simplified this procedure by collecting larger volumes of buffer at rotor speeds greater than those required for basophil elution (>2350 rpm) thereby

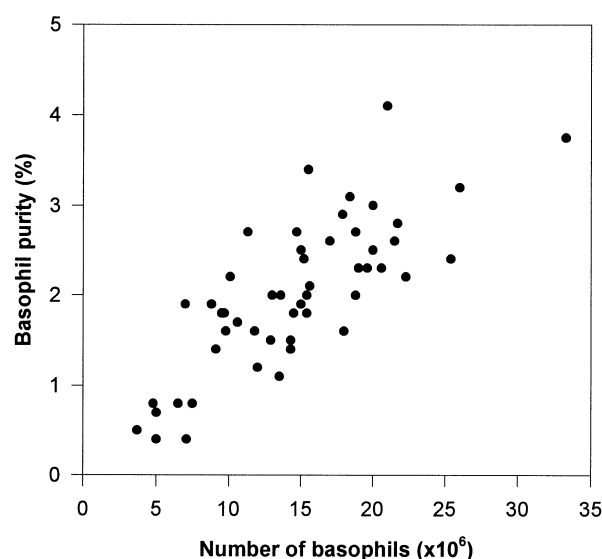


Fig. 1. Relationship between basophil number and purity following initial enrichment using Ficoll density centrifugation ($n = 81$; $r^2 = 0.53$). Basophils were stained with alcian blue stain and counted using a haemocytometer (Fuchs-Rosenthal). The percentage purity was assessed by dividing the number of basophils with the total number of nucleated cells $\times 100$. A clear correlation can be seen and demonstrates the large variation between donors.

holding back the basophils in the chamber. The limitations of elutriation for basophil enrichment probably lies not only in the differing densities of basophil populations but on aggregation of the cells in the elutriation chamber. In order to overcome conglomeration of cells in the elutriation chamber it was sometimes necessary to briefly apply pressure to the inlet tube, which disrupted these cell aggregates.

Step 3: Immunomagnetic beads. The final basophil purity obtained after this stage was $67.3 \pm 1.4\%$ and a mean yield of $3.5 \pm 0.5 \times 10^6$ basophils ($n = 51$). Figures 2 and 3 summarise the results obtained during the basophil enrichment procedure in terms of purity and yield respectively. It is clear from these results, that the purity and yield is very donor dependent. Cytospin preparations from highly enriched basophils show that these cells exhibited normal morphology under a light microscope (Fig. 4). Purified basophil preparations were found to be $97.7 \pm 1.1\%$ viable using the Trypan blue exclusion test.

In 10 experiments, the third step, with immunomagnetic beads, was repeated in order to establish whether the purity could be increased. Basophil preparations in these experiments were $44.0 \pm 5.1\%$ pure with a yield of $4.3 \pm 0.8 \times 10^6$ basophils following the first enrichment with immunomagnetic beads and $59.0 \pm 5.2\%$ pure with a yield of $3.4 \pm 0.8 \times 10^6$ basophils after a second incubation. Thus, for experiments where a poor basophil purity was obtained these could not be adequately enriched further without a substantial loss in yield. Additionally, basophil preparations of more than 85% pure following the immunomagnetic bead step were not strikingly purified further (maximally to 90% purity) thus demonstrating limitations of the negative selection procedure in trying to obtain homogeneous

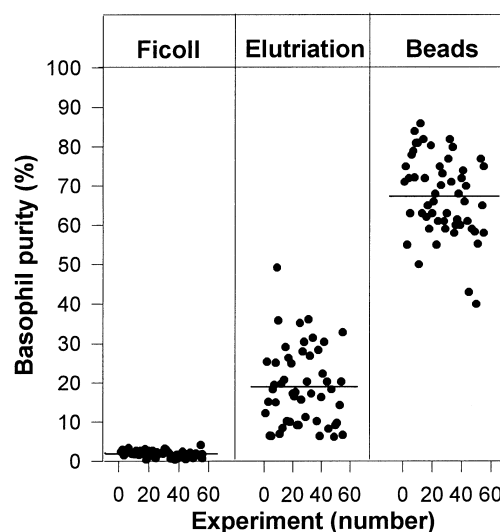


Fig. 2. Basophil purity obtained from 51 donors following Ficoll density centrifugation (Ficoll), counter current centrifugal elutriation (Elutriation) and the final purity following negative selection by immunomagnetic beads (Beads). Bars depict mean values.

basophil preparations containing large numbers ($>1 \times 10^6$) of the cells.

In seven different experiments, Percoll gradients were used following the elutriation step according to the method described previously [8, 9]. These preparations were $18.4 \pm 2.3\%$ pure following elutriation. However, following Percoll enrichment, the purest basophil fractions gave rise to a mean purity of $54.0 \pm 7.4\%$ and a substantial overall loss of recovery. Furthermore, these cells were not consistently enriched further by immunomagnetic beads (mean final purity: $60.4 \pm 9.3\%$). The results obtained from the observations using a second immunomagnetic bead step as well as Percoll were not included in Figures 2 and 3.

The ability of certain basophil subpopulations to express CD16 was also investigated in terms of the possible loss of these cells during the negative selection step using immunomagnetic beads. However, immunomagnetic beads directed against CD16 did not consistently bind to basophils and, maximally, less than 0.5% of total basophils were lost.

Histamine release

Total basophil histamine content from 15 donors was 1.60 ± 0.08 pg/cell following Ficoll, 1.54 ± 0.10 pg/cell after elutriation and 1.55 ± 0.13 pg/cell following final purification. The larger histamine content measured in less pure preparations may have partially arisen from contaminating cells such as eosinophils, which also store the amine. Additionally, mean spontaneous histamine releases from these donors were $4.2 \pm 0.8\%$ after Ficoll, $5.1 \pm 1.2\%$ after elutriation and $5.3 \pm 1.3\%$ for the final purified basophils.

Purified basophils were highly responsive to stimulation with anti-IgE (Fig. 5) and the calcium ionophore A23187 (Fig. 6). Although no significant differences could be measured when comparing these preparations to those from the Ficoll or elutriation step there was a slightly

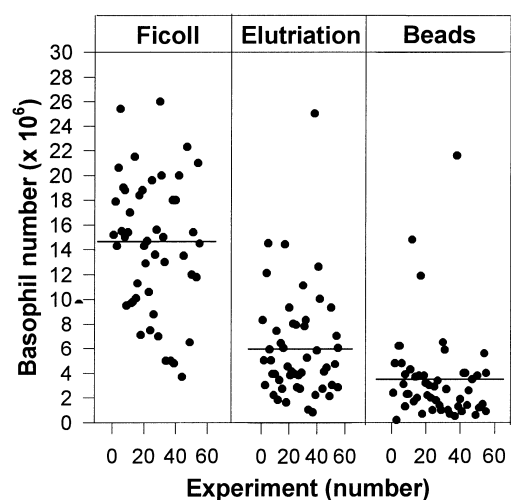


Fig. 3. Basophil number obtained from 51 donors following Ficoll density centrifugation (Ficoll), counter current centrifugal elutriation (Elutriation) and the final purity following negative selection by immunomagnetic beads (Beads). Bars depict mean values.

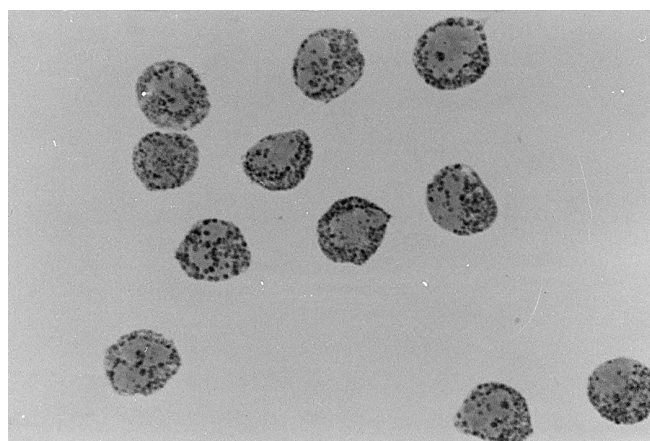


Fig. 4. Purified basophils stained with May-Grünwald (magnification $\times 359$).

reduced histamine release from the purified cells at maximal concentrations of both agonists.

We observed no correlation between basophil histamine releasability and IgE levels in sera from these donors. In addition, no correlation was observed between sera IgE levels and either basophil number or purity at any stage of the enrichment procedure. In 3 out of 15 experiments where histamine release was measured, were found to be non-responders to anti-IgE stimulation. However, 2 of the 3 non-responders did release large amounts of histamine due to calcium ionophore A23187.

Electron microscopy

Basophils obtained from the counter current centrifugal elutriation process and after the final purification step with immunomagnetic beads showed no signs of cellular activation as determined by electron microscopy (Fig. 7). From

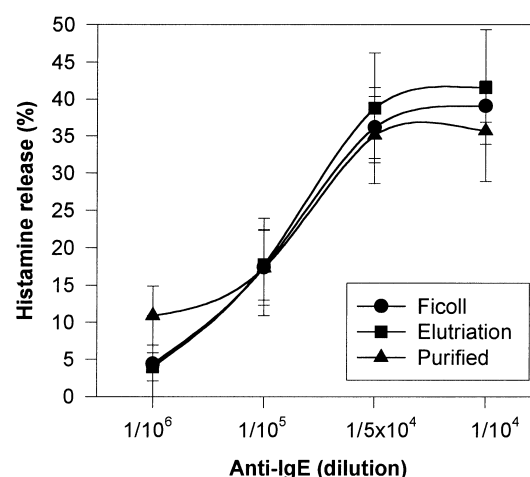


Fig. 5. The effects of purification on anti-human IgE induced histamine release from human basophils ($n = 12$). Spontaneous histamine releases were $3.9 \pm 0.9\%$ following Ficoll density centrifugation, $5.7 \pm 1.5\%$ after counter current centrifugal elutriation and $6.2 \pm 1.22\%$ for the final purified basophil fractions.

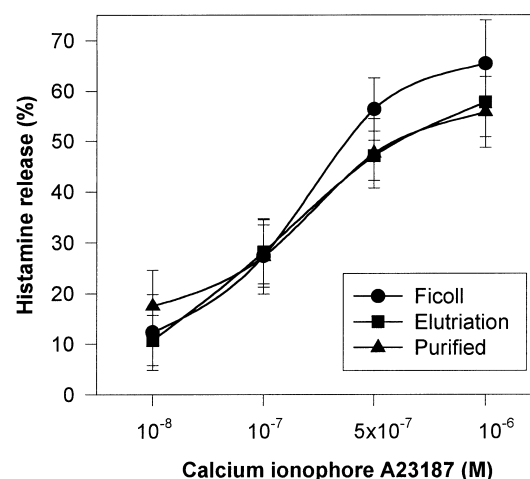


Fig. 6. The effects of purification on calcium ionophore A23187 induced histamine release from human basophils ($n = 12$). Spontaneous histamine releases were the same as those shown in Fig. 5.

these studies, the cells appeared rounded, with polylobed nuclei containing heavily condensed chromatin within the nuclear envelope. On the surface membrane, the basophils showed short, blunt and irregularly distributed processes. Granules were filled with electron-dense material enmeshed in a less dense matrix. There were no signs of degranulation.

Discussion

The results shown in this paper clearly demonstrate the large donor variations observed during basophil purification. The considerable number of publications regarding basophil purification does indicate that the problems of donor variation have to be assessed more comprehensively. In our studies, although we were able to enrich basophils to 75–86% purity in 14 out of 51 donors, a large number of

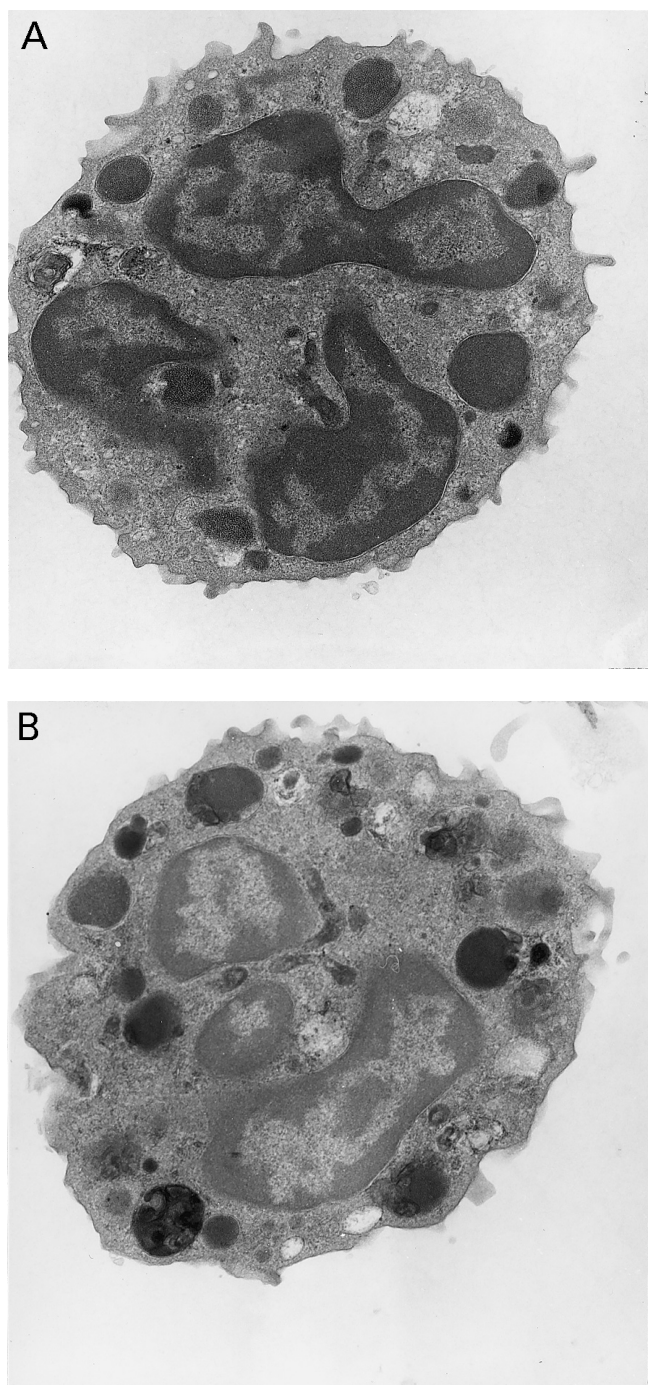


Fig. 7. Electron microscopy of human basophils following counter current centrifugal elutriation (A) and final purity after negative selection with immunomagnetic beads (B).

experiments produced considerably lower purities. However, we were able to reach higher yields of basophils than those obtained by other authors although these high yields do appear to compromise the final purity obtained using immunomagnetic beads. This is because semi-purified basophil fractions of greater yield also contain proportionally larger numbers of other cell contaminants, making their removal by negative selection less successful and costly due to cell aggregation and numbers of immunomagnetic beads

required. We suggest that this compromise of yield and purity together with the donor variation observed following the first enrichment by Ficoll is the main limitation encountered with the enrichment of basophils using our method.

By far the easiest method of purification, and also guaranteeing high purity, is that of positive selection. A recent publication by Wilhelm et al. [11] used positive selection of basophils by mAb to Cdw17 as did Weil et al. [12], Graziano et al. [13] and MacGlashan et al. [16] via selection of the IgE receptor. However, these methods, though resulting in high purity, give rise to substantially lower histamine release induced by immunological activation and altered cell function compared to unpurified preparations. It is therefore questionable, whether any method that uses positive selection of the cells can exclude the likelihood of cell activation and the consequent functional differences arising from it.

The use of negative selection by immunomagnetic beads has been speculated to indirectly activate basophils [10]. However, given the reliability of this technique compared to Percoll gradients for basophils, we have not found any superior alternative. Furthermore, cells incubated with immunomagnetic beads were in Ca^{2+} -free buffer at 4°C so making the likelihood of indirect basophil activation remote. Kepley et al. [10] circumvented the need for immunomagnetic beads by the use of cell-sorting, which gave rise to highly pure basophil populations. However, this technique does not appear to suit basophil purification when large numbers of the cells are required.

We observed that the use of Percoll gradients produced inconsistent results and was therefore not employed in our method. A recent paper by Bjerke et al. [9] reported that Percoll purification of basophils was only successful using freshly drawn blood due to density changes with storage time. However, this method appears to be cumbersome as a first step when large numbers of enriched basophils are required and the use of buffy coat blood excluded. Furthermore, the high purities reported by these authors following negative selection by immunomagnetic beads was achieved with a low number of basophils.

Elutriation was found to be the most reliable and swift enrichment step. However, it was difficult to reproduce the technique with different elutriators of the same category without modifying the speed. We also found that system pressures differed according to the elutriation chamber types used and learned that this variation may arise since some of these components are individually constructed by hand. Furthermore, oral communications from Mr. Odenthal (Beckman Instruments) confirmed that variations, of up to 20%, may ensue in terms of the flow rates or centrifugal speeds employed in order to obtain the same result for a particular cell type (or standardized test particle) to be eluted between different elutriators. This problem must therefore be taken into account when establishing elutriation methods and the speed and flow rates adjusted accordingly.

Functional studies showed that there were no significant differences between histamine release from basophils of varying purities induced by anti-IgE or calcium ionophore A23187. However, the slight flattening of the dose-response curve observed with purified basophils stimulated with anti-IgE in some experiments may indicate a loss of receptor

bound IgE, which would explain the greater release observed at lower concentrations of this stimulus. Although, a similar pattern of response was also observed with the calcium ionophore A23187. Given the evidence from electron microscopy studies which show no morphological change, this suggests that highly purified basophils may display a down-regulated signal transduction that possibly arises due to the lack of priming factors from other cell contaminants. Morphological evidence, as shown by electron microscopy, also demonstrated that no anaphylactic or piecemeal degranulation of the cells had occurred.

We therefore conclude that the techniques employed in our study for the enrichment of basophils maintain unimpaired cell function and give rise to high yields of the cells. However, there still exists a need for the attainment of high yields of homogeneous basophil populations. The parameters of existing techniques must be defined for a wide range of donors and further studies are vital.

Acknowledgements. The authors would like to thank Claudia Albrecht, Achim Gronow, Heike Kühl and Gisela Brinkmann for excellent technical assistance. We would also like to thank the Deutsche Forschungsgemeinschaft (grants AM 79/2-1 and HA 1590/2-1), the Basotherm Stiftung e.V. and the Royal Society (London).

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