Purification of human basophils by density and size alone

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Abstract:

Basophils typically account for approximately 1% of the white cells in peripheral blood. We have developed a unique method for purifying basophils from whole blood of normal subjects to at least 95% purity. Basophils are separated from other cell types dy density-dependent sedimentation in Percoll and cell sorting, based solely on their size and granularity. The mean overall yield ranged from 5% to 28%. The procedure is typically completed within 4 h. The highly purified basophils obtained are functionally competent and morphologically intact. They release histamine in response to FceRI-mediated stimulation, express FceRI and BSP-1 ligand as analyzed by flow cytometry, and exhibit the known characteristic ultrastructural features of basophils by electron microscopy. This procedure avoids positive-selection antibodies that might perturb receptors on basophils or negative-selection antibodies that might activate other cell types, and can be used to obtain basophils for studies in vitro.

Keywords: Basophil | Flow cytometry | Electron microscopy | FceRI | Histamine | Purification

Article:

1. Introduction

Basophils and mast cells are thought to play important roles in diseases involving IgE-mediated, immediate-type hypersensitivity due to high affinity receptors for IgE (FceRI) on their cell surfaces and histamine in their secretory granules (Schwartz and Huff, 1993; Ishizaka et al. 1972).

The precise involvement of the basophil in the early-phase and late-phase response to allergens remains poorly defined because no marker exists with adequate sensitivity and specificity to detect basophils in tissues or evidence for basophil activation in blood. The low numbers of basophils in the peripheral blood greatly limit their availability for study. Several procedures for the isolation of human basophils from peripheral blood have been described (MacGlashan, Jr. and Lichtenstein, 1980; Schroeder and Hanrahan, Jr., 1990; Bodger and Newton, 1987; Mulet al., 1992; Tanimoto et al., 1992; Bjerke et al., 1993). These include one-step gradient centrifugation which usually results in purities under 50%; positive immunoselection with IgE and either anti-IgE antibody or antigen; and negative selection with antibodies that selectively recognize other cell types, such as CD2 on T lymphocytes, CD19 on B

lymphocytes, and CD14 on monocytes. Positive selection methods for sorting basophils that bind to and possibly cross-link FceRI may modify the functional properties of basophils, and co-purify unwanted B lymphocytes expressing membrane IgE or activated eosinophils and macrophages expressing FceRI (Gounni et al., 1994; Wang et al., 1992; Bieber et al., 1992). Negative selection methods, though typically performed at low temperatures to suppress signal transduction, may still activate other cell types if experimental conditions become permissive, for example, during the sorting itself.

Our goal was to obtain pure populations of basophils without using immunological reagents that are costly and might affect basophil function. To this end, a unique method was developed for the purification of basophils from whole blood (0.5% to 2% purity) to > 95% purity in less than 4 h. This method takes advantage of the unique granularity and size of the basophil.

2. Materials and methods

2.1. Materials

Percoll, CPSR-3 (controlled processed serum replacement-3), ethylenediaminetetraacetic acid EDTA, FITC-conjugated anti-mouse IgG and IgM, RPMI 1640, normal human AB serum, bovine serum albumin (BSA), non-specific mouse IgG1 (MOPC31C) and IgM (MOPC 104E), try-pan blue and propidium iodide (Sigma Chemical Co., St Louis, MO); Isoton II balanced electrolyte solution (sheath fluid) (Coulter Diagnostics, Hialeah, FL); Hanks' balanced salt solution (HBSS), HBSS without Ca 2+ or Mg 2+ (HBSS =) (Gibco, Grand Island, NY); CLB-CDw49b (murine monoclonal IgG1 anti-VLA-2) (Research Diagnostics, Flanders, N J); and Wrights stain (Fisher Scientific, Norcross, GA) were obtained as indicated. BSP-1, an IgM murine monoclonal antibody recognizing a basophil surface antigen (Bodger et al., 1987), was provided by M.P. Bodger (Christchurch Hospital, New Zealand). The mouse monoclonal antibody YB5.B8 (IgG) recognizes Kit (Gadd and Ashman, 1985; Mayrhofer et al., 1987; Lerner et al., 1991), the cell surface receptor for the ligand termed stem cell factor (also Kit ligand, Steel factor or mast cell growth factor) and was provided by L.K. Ashman (University of Adelaide, Adelaide, Australia). The a chain of the high affinity receptor for IgE (FceRI) is recognized by the IgG mono-clonai antibody termed 29C6 (Riske et al., 1991), and was provided by R. Chizzonite (Hoffman LaRoche, Nutley, N J).

2.2. Purification of human basophils

Venous blood was collected from normal volunteers who had given informed consent as approved by the Human Studies Committee at Virginia Commonwealth University. None were atopic by history. Blood specimens (50 ml) containing 0.5% to 2% basophils were anticoagulated with 0.01 M EDTA, diluted 1/1 with 1 × HBSS=and 12 ml layered onto 12 ml of 45% (density 1.070 g/ml), 12 ml of 58.5% (density 1.080 g/ml), and 12 ml of 65% (density 1.09 g/ml) Percoll, prepared essentially as described previously (Leonard et al., 1984). Densities were confirmed by refractometry using an American Optical refractometer (Scientific Instruments, Keene, NH). After centrifugation at 700 × g for 15 min at room temperature in a DPR 6000 centrifuge (IEC, Needham Heights, MA) with a Model 269 swinging bucket rotor, basophils were collected predominately from the 45%/58.5% interface at purities ranging from 20% to

45%. Percoli-enriched cells were washed three times with phosphate-buffered saline (PBS) containing 1% CPSR-3 and 0.01 M EDTA at 4°C, conditions designed to prevent basophil activation, and were then subjected to flow cytometry and cell sorting on an Epics 753 flow cytometer (Hialeah, FL). Sorting was performed at a flow rate of 500-1000 cells/s and a sort rate of 100-200 cells/s. Basophils were collected into 1.5 ml centrifuge tubes containing PBS with 1% CPSR-3 and 0.01 M EDTA. Viability of the sorted cells was assessed by trypan blue exclusion.

2.3. Analysis by flow cytometry

Sorted basophils were washed and resuspended in RPMI 1640 with 10% normal human AB serum to a concentration between 5.0 × 106 and 9.0 × l06 cells/ml at 4°C for 30 min. Samples were centrifuged at 400 × g for 5 rain and washed with PBS containing 1% BSA and 0.1% sodiumazide (wash buffer). Cells were incubated with primary experimental and isotype-matched control antibodies at concentrations indicated (see results section) for 30 min at 4°C. After washing three times with wash buffer, the cells were incubated with a 1/50 dilution of fluorescein isothiocyanate (FITC)-labeled secondary antibodies against mouse IgG and IgM immunoglobulins for 30 min at 4°C. Samples were washed as above. Cell pellets were resuspended in 200 μl of sheath fluid. Just prior to reading samples, propidium iodide (5 p.g/ml, final concentration) was added to each sample to detect and exclude dead cells from the analysis. Dead cells typically accounted for < 5% of total cells. Flow-cytometry was performed in a FACScan flow-cytometer (Becton and Dickinson, San Jose, CA). Cells were gated according to their forward and side scatter, and were analyzed for binding of primary antibodies by fluorescence intensity with CyCLOPS software (Cytomation, Fort Collins, CO).

2.4. Histamine release

Purified basophils (50000 cells/aliquot) were washed two times with Tyrode's buffer containing gelatin (0.1% w/v) without calcium or magnesium (TGD =) and then resuspended in 100 μl TGD (containing both calcium and magnesium) with 0.38 μg/ml to 3.0 μg/ml of 29C6 at 37°C. After incubation for 30 rain the ceils were centrifuged at 900×g for 5 min at 4°C in a DPR 6000 centrifuge. Supernatants were collected and the cell pellets were resuspended in 100 μl of TGD = buffer. All cell pellets were sonicated with 10 pulses (power setting #4, 50% pulse cycle, microtip attachment) from an Ultrasonics sonicator (model w-225, Ultrasonics, Plainview, NY) and centrifuged at 12000 ×g for 5 min at 4°C in a Beckman 12 microfuge (Palo Alto, CA). Histamine was measured by a radioimmunoassay (AMAC-Immunotech, Westbrook, ME). For measurement of the total histamine, 50000 unstimulated cells were lysed by sonication as above and centrifuged at 12000 ×g for 5 rain at 4°C to remove debris; and the supernatant was collected for analysis. Spontaneous histamine release was determined by incubating cells in TGD without stimulatory antibody. All percent release values were corrected for spontaneous release and calculated as:

net % release
= {(stimulated supernatant)- (spontaneous supernatant)}
/{(stimulated pellet) + (stimulated supernatant)
- (spontaneous supernatant)} ~ × 100

2.5. Electron microscopy

Sorted basophils were centrifuged at 400 ×g for 5 min and the supernatant removed. These ceils were fixed for 90 min at 4°C in modified Karnovsky's fluid (1% glutaraldehyde, 3% paraformaldehyde and 4% sucrose in 0.1 M cacodylate buffer, pH 7.2). The ceils were washed overnight in cacodylate buffer containing 5% sucrose (three changes), postfixed in 1% osmium tetroxide in the same buffer with 0.5% sucrose and then dehydrated through graded ethanols and propylene oxide. The ceils were embedded in araldite, Fluka Durcupan ACM. Thin sections (60-90 nm) were cut on a Sorvall-Dupont Ultra-microtone and collected on copper grids. Sections were stained with uranyl acetate (Watson, 1958) and lead acetate (Reynolds, 1963) and examined with a JEM 1200 EX electron microscope.

3. Results

3.1. Basophil purification

The purity of basophils in whole blood ranged from 0.6% to 2%, and starting basophil numbers were estimated to range between 2×10^6 and 12×10^6 (Table 1). Basophils in whole blood were partially purified by density-dependent centrifugation in Percoll to purities ranging from 20% to 45% basophils. The estimated yield after Percoll was about 50%. Percoll-enriched basophils were contaminated mostly with monocytes and lymphocytes as determined by microscopic inspection of cytospin preparations stained with Wrights stain.

Basophils were further purified by cell sorting with a yield from Percoll-enriched to sorted basophils of 23 + 4.5% (mean + SD) (Table 1). A typical flow cytometry pattern of Percoll-enriched peripheral blood basophils is shown in Fig. 1. Basophils appear at a slightly larger size (forward scatter) and density (side scatter) than the mononuclear cells and red blood cells, but at a lower density than neutrophils and eosinophils (data not shown). Cells in the gated region labeled b in Fig. 1 were collected and found to contain 98% basophils by staining with Wright's stain (Fig. 2). Viability was greater than 95% in this experiment and in the other four purifications by trypan blue exclusion. Final concentrations were consistently >_ 95% (Table 1). These cells also stained with Alcian blue and exhibited metachromasia when stained with toluidine blue, but were not stained by alkaline phosphatase-conjugated anti-tryptase antibody (G3) (not shown), a selective marker for human mast cells (Irani et al., 1989). The population of cells just below the gate, when examined, primarily included basophils, mononuclear cells and erythrocytes. In Fig. 1 these cells were collected and consisted of 66% lymphocytes/monocytes, 7% basophils, and 27% erythrocytes.

3.2. Flow cytometry

The purified basophils were further examined by flow cytometry for various surface antigens as shown for one preparation in Fig. 3. Two separate preparations were examined, each giving comparable results. BSP-l-labeled basophils exhibited a multiphasic pattern, with 29% overlapping with the negative control (stained with irrelevant mouse IgM), the remainder (71%) being positive, most of these residing in a broad peak between 5 and 70 MIF. Anti-FceRIa-labeled cells also showed a multiphasic distribution; the majority (53%) in a peak with a MIF of 122 (99-167 MIF), another group (19%) between 39 and 97 MIF, and a third group (20%) in a

smaller peak at a MIF between 1-38. A few ceils appeared at a MIF higher than 167. Thus, approximately 80% of the cells were positive for 29C6.

Kit, which is known to be strongly expressed on mast cells and negative or weakly expressed on basophils, also was examined (Fig. 4C). As anticipated, a small unimodal shift in the cells was observed when labeled with YB5.B8, consistent with small amounts of Kit being available on the surface of these cells, though only 13% of the labeled cells were outside of the range for the negative control. Also, one basophil preparation was negative with anti-VLA-2 antibody, a marker for platelets, suggesting that substantial platelet contamination was unlikely. The pattern of surface marker expression is consistent with the purified cells being basophils.

3.3. Basophil ultrastructure

Ultrastructural analysis of the sorted cells was performed by transmission electron microscopy. A typical cell with the characteristic features of human basophils is shown in Fig. 4A. These include relatively short, thick extensions of the plasma membrane, and large cytoplasmic granules. Granules exhibit a range of patterns and electron densities with most exhibiting particulate material, and some also displaying myelin-like, particle-filled circles and homogeneous electron-dense regions (Figs. 4B,4C and 4D). These cells were morphologically indistinguishable from presorted basophils (not shown). Also, no evidence for platelet contamination was detected during this ultrastructural analysis.

3.4. Basophil activation

The ability of purified basophils to release histamine was examined using an anti-FceRIa-chain antibody to stimulate exocytosis. The amount of histamine measured was 1.2 ± 0.11 $\mu g/10^6$ basophils (mean $\pm$ SD, n = 2), which is comparable to published values (MacGlashan, Jr. and Lichtenstein, 1980; Schroeder and Hanrahan, Jr., 1990). Results in Fig. 5 show a dose-dependent net% release of histamine ranging from 11% at 0.38 μg 29C6/ml to 70% at 3 μg 29C6/ml that is FceRI-mediated. Spontaneous release was < 5% in each experiment. In another experiment, basophils were challenged before (28% purity) and after (98% purity) cell sorting. The net % release values were 54 and 57 (spontaneous release < 5%) at 2 μg 29C6/ml. Histamine content of basophils in this experiment was calculated as 1.1 $\mu g/10^6$ and 0.8 $\mu g/10^6$ basophils, respectively. The new values obtained are comparable to those in Fig. 5 and suggest that sorting does not select for a more activatable population of basophils. Thus, these highly purified preparations of Percoll-enriched and sorted basophils are functional with respect to FceRI-mediated degranulation.

4. Discussion

The method for the purification of human basophils described in the current study uses density-dependent sedimentation in discontinuous Percoll gradients and cell sorting by density and size-dependent flow cytometry. This procedure offers four advantages over others used to isolate basophils. First, the basophil membrane is not directly perturbed with antibody during the purification procedure, because no antibodies are used for positive selection. Second, negative selection is avoided. Activation of other cell types that might influence basophils is thus not a concern. Three, special immunologic reagents are not needed. Fourth, the procedure can be

performed in about 4 h, taking basophils in 50 ml of blood from 1% to at least 95% purity with a total yield estimated to range from 5% to 28%.

Analysis of sorted cells by flow cytometry for surface markers previously shown to be present on human basophils further supports their identity as basophils (Fig. 3). Cells analyzed for FceRI showed a multiphasic distribution, suggesting variability in the amounts of this receptor for IgE that is available to bind anti-a-chain antibody. Basophils also were found to Express small amounts of surface Kit. Although an early study did not recognize this low level of Kit (Ashman et al., 1986), a more recent study did (Columbo et al., 1992).

The functionality of basophils purified in the current study was established by showing a dose-dependent release of histamine in response to anti-FceRIa antibody, which cross-links FceRI receptors. The maximal net histamine release of 70% obtained here is somewhat higher than values of 16-50% typically reported using anti-IgE antibodies (Ishizaka et al., 1972; MacGlashan, Jr. and Lichtenstein, 1980; Schroeder and Hanrahan, Jr., 1990; Mul et al., 1992; Riske et al., 1991; Tanimoto et al., 1992; Bjerke et al., 1993). The magnitude of degranulation presented here is more similar to that induced by A23187 and f-Met-Leu-Phe (Mui et al., 1992). Whether this apparent higher immunologic releasability is due to the milder purification conditions used in the current study will need further clarification.

Purified basophils by electron microscopy showed no evidence of damage, and no evidence of anaphylactic degranulation or piecemeal degranulation (Fig. 5), which are known to occur with basophil activation (Dvorak, 1988; Hastie, 1990; Dvorak et al., 1992). Granules showed varying degrees of electron density with occasional myelin-like or electron-dense figures over a particulate background. These granules are clearly distinguishable from the scrolls, gratings and lattices described for granules of mature mast cells (Craig et al., 1988; Dvorak, 1988), and from the dense core type structures in the smaller granules of immature skin-derived mast cells (Craig et al., 1989). Plasma membranes exhibited relatively short, thick extensions; in contrast, mast ceils have more numerous extensions that are long and thin. These features of the purified cells described in the current study together with their nuclei with deeply divided lobes are consistent with these cells being mature, unstimulated human basophils.

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