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Isolation of Human Basophils

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Abstract

Human basophils are among the rarest of all circulating leukocytes (typically ~2–8×10^4/ml of blood), which has long complicated their isolation from blood. In this unit, we describe a versitle approach whereby basophils can be rapidly isolated from blood leukocytes as enriched suspensions (5%–25%) using density centrifugation. Alternatively, combining this enrichment step with negative selection using immunomagnetic beads allows for the preparation of suspensions exceeding >99% basophils. Depending on needs, either approach allows for a variety of *in vitro* assays investigating basophil mediator release and/or cytokine secretion.

CAUTION—When working with human blood, cells or infectious agents, biosafety level 2 practices should be followed (see Chapter 7 introduction).

Keywords

Immunologic Studies in Humans; In Vitro Assays for Immune Cell Function; Innate Immunity; Immune Disease

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BASIC PROTOCOL

BASOPHIL ENRICHMENT BY PERCOLL GRADIENT CENTRIFUGATION

Materials

Venous blood, freshly drawn and preferably into EDTA (purple-top vaccutainer tubes)

Note: Drawing blood into 60 cc syringes pre-loaded with 5 ml 0.1 M EDTA, pH 7.5 works well when collecting volumes 50cc. This is achieved by using a shielded butterfly needle (19G) with 12″ tubing (Becton Dickinson), which is pinched-off and transferred between multiple syringes during blood drawing.

Percoll gradients in 50–ml polystyrene conical tube (see *SUPPORT PROTOCOLS*)

PIPES/albumin/glucose buffere (PAg; without Ca^{++}, Mg^{++}, and phenol red; *APPENDIX 2*)

50–ml polypropylene conical tubes

Centrifuge with rotor for swinging bucket carriers and manual slow start-up feature (e.g. tabletop model HN-SII, International Equipment Company –IEC or equivalent)

3–ml disposable polypropylene transfer pipets

15–ml polystyrene conical tubes

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Additional reagents and equipment for blood collection and for Alcian blue staining (see *SUPPORT PROTOCOLS*)

Standard Operating Procedure (SOP) per 50cc blood volume

1. After mixing well by inverting 10–20 times, divide each 50 ml portion of EDTA blood between 2–50–ml polypropylene conical tubes. Centrifuge 15 min at 300 × g, room temperature (~23°C), with no brake.

2. Carefully remove much of the plasma without disturbing the buffy-coat layer on top of the red cell pellet. Then, using a 3 ml bulb transfer pipet, carefully aspirate the buffy-coat layer, transferring to another 50ml tube containing 20 ml PAG-EDTA buffer. Three to four separate aspirations are usually enough to collect entire buffy-coat interface. Two buffy-coat interfaces are pooled into the same 20ml of PAG-EDTA, giving a final volume of 40–45ml of diluted buffy-coat.

   Note: Many red cells are also aspirated during this step to ensure recovery of buffy-coat (and basophils). By excluding plasma and diluting buffy-coats in PAG-EDTA buffer, much of the variability in plasma content among subjects (e.g. fat) is eliminated and thus less likely to alter density centrifugation.

3. Using a 3-ml bulb pipet, carefullylayer 20 ml of well-mixed diluted buffy-coat over each Percoll gradient and centrifuge 20 min at 1600 rpm (700 × *g*), room temperature, with no brake. When starting centrifugation, it is critical to start the rotor slowly to prevent admixing of the Percoll gradients.

4. Gently remove the gradients from the centrifuge. If the upper interface of cells (i.e. Basophil-depleted cell, BDC, fraction) is to be discarded, this is best done using an aspirator with a 200ml pipet tip connected to the hose and slowly encirciling the perimeter of the tube at the interface level. Alternatively, BDC can be collected, washed free of platelets (e.g. 4× washes at 150 × g), and used much like PBMC, only depleted of basophils and some mononuclear cells. The basophil-enriched cell (BEC) fraction is carefully removed using a 3 ml bulb transfer pipet, pooling interfaces from two gradients into a 50 ml conical tube containing ~15 ml PAG-EDTA and pre-coated to prevent cell sticking to the plastic. Cells are washed in a total volume of 40 ml and centrifuged for 10 min. at 150×g.

   In harvesting the BEC fraction from the Percoll gradients, the total volume taken is approximately 10 ml (i.e. the bottom half of the 1.072 g/ml Percoll, the BEC interface, and the top half of the 1.081 g/ml Percoll).

5. After discarding the 40 ml wash fluid, the cell pellet is resuspended in ~5 ml PAG-EDTA, resuspended and transferred to a pre-coated 15 ml conical tube. The 50 ml tube is rinsed with another ~5 ml of buffer before pooling into the 15 ml tube and centrifuging at 4°C, as above.

   If basophilsare to be further purified, see protocol below.

6. After discarding the ~10 ml wash fluid, the BEC pellet is resuspended in ~5 ml of cold PAG (without EDTA) and once again centrifuged at 4°C, as above.

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7. After discarding the wash fluid, the BEC cell pellet is carefully resuspended in 0.5 ml cold PAG and counted using Alcian Blue staining (see *SUPPORT PROTOCOLS*). Cells are kept on ice until ready to use.

   Basophil numbers and percentages can also be estimated by: 1) determining the total cell count of the BEC suspension using a standard hemacytometer and 2) making a cytospin with ~5×10^4 total cells and staining with Wright's stain. Most basophils appear with intracytoplasmic granules stained dark reddish purple.

Basophil Purification Using Negative Selection with Immunomagnetic Beads —There are now commercial kits that enable further purification of basophils using negative selection protocols, which essentially label all contaminating cells with specific antibodies for removal on magnetic columns. In fact, it has long been our experience that when used in conjunction with the Percoll density centrifugation enrichment step, these kits allow for the preparation of basophil suspensions that routinely exceed >99% purity, with yields ranging from 50–100%. Until recently, we have employed the StemCell Technologies antibody cocktail and beads (cat. #14059C) along with LS columns (cat. # 130-042-401) from Miltenyi Biotec. However, with the StemCell kit no longer sold, but through special order, we have more recently converted to the antibody cocktail and bead kit from Miltenyi (cat. #130-092-662).

Standard Operating Procedure (SOP) for purifying basophils from BEC suspensions

1. Rather than proceeding to the PAG wash, as described in step #5 above, the BEC pellet is resuspended in column buffer (5μl per 1×10^6 total cells). Antibody cocktail is immediately added (0.25μl per 1×10^6 total cells) and incubated 30 min. on ice.

   NOTE: Package insert protocols generally state using 4× more antibody cocktail (e.g. 1 μl per 1×10^6 total cells). However, these SOPs are based on using PBMCs prepared on single Ficoll gradients (1–3% basophils), which means many more contaminants, particularly monocytes and lymphocytes, need to be removed in order to achieve basophil purification. BEC suspensions showing neutrophils exceeding 20% or with heavy red blood cell contamination, may also require antibody cocktail amounts closer to those recommended in the package insert.

2. Microbead reagent is then added, usually at 70–80% the amount of antibody cocktail used. The suspension is gently mixed and incubated for 30 min. on ice. Gentle mixing after 15 min. is recommended to prevent excessive clumping of cells and beads.

3. Column buffer (5–8 ml) is added and the suspension gently mixed before centrifuging at 150×g for 10 min., 4°C. During the centrifugation step, an LS column (attached to a MidiMax or QuadraMax magnet) is primed with column buffer (3–5 ml).

4. Upon discarding the wash fluid, the cell pellet is resuspended in 0.5–1.0 ml column buffer and gently mixed by pipetting up and down ~20 times. Then, the entire suspension is applied to the primed column, with a 15 ml collection tube in place

on ice to collect basophils. An equal volume of column buffer is immediately used to rinse residual cells from the incubation tube, with this also applied to the column.

5. Once the flow-thru is complete, the column is rised with ~3 ml column buffer and this also collected. Upon completion, the collection tube is removed from the ice and centrifuged for 10 min. at 150×g. A subsequent wash in cold PAG (5–8 ml) is then done before resuspending the final cell pellet in PAG (0.5–1.0 ml) and counting for basophils using Alcian Blue staining.

SUPPORT PROTOCOLS

PERCOLL GRADIENT PREPARATION

Additional Materials

Percoll (GE Healthcare Bio-Science AB, Uppsala, Sweden)

10× PIPES buffer (prepared as a 10-fold higher concentration stock solution containing 250 mM PIPES (Sigma Chemical Co., cat. #6757), 1.10 M NaCl, 50 mM KCL in double-distilled (dd)H_2O. *without* Ca^{++}, Mg^{++}, and phenol red) Slowly pH to 7.3 with **10 N NaOH**, bring to final vol. with ddH_2O, and filter (Whatman #1).

Note: PIPES is the free-acid form and will only go into solution completely once pH to 7.3 –do not deviate from the reagents cited! Keep at 4°C for <1 month.

1× PIPES buffer (made by mixing 1 part 10× PIPES with 9 parts ddH_2O). Stored at 4°C.

PAG buffer (made as if preparing 1× PIPES, but additionally containing 0.003% human serum albumin (Calbiochem-Behring Corp.) and 0.1% D-glucose. Stored at 4°C.

PAG-EDTA (made as if preparing PAG, but also containing 4mM EDTA). Stored at 4°C.

Column buffer (made as if preparing 1× PIPES, but also containing 1% bovine serum albumin (BSA) and 2mM EDTA. Stored at 4°C.

9″ pastuer glass pipets with small opening at narrow-end tip

Made by heating over a blue flame of bunson burner and pulling on both ends of pipet. Small opening (~0.1mm) at pipet tip made by gently clipping with scissors

50–ml polystyrene conical tubes (AccuSpin tubes from Sigma Chemical, optional)

1. Prepare an isotonic Percoll stock solution (IPS) in a 500–ml container by mixing the following:

   450 ml Percoll

   50 ml 10× PIPES

   The pH of the resulting solution should be ~7.4. Prepare less IPS if not used within 2 weeks

Approx. Density (g/ml)	Isotonic Percoll solution/1× PIPES (% IPS) (ml/ml)
1.075	14.00:11.40 (~55%)
1.081	15.50:9.75 (~61%)

2. Carefully prepare the above solutions, which is enough for 2 gradients:

Prepare the above 55% and 61% solutions the day of the experiment (2 gradients per 50 ml specimen of blood). Prepare more or less accordingly, depending on amount of specimen. Although not necessary, density of the solutions can be verified using a density meter or by measuring refractive index (RI) at 22°C with a refractometer. The HSA and EDTA in the PIPES-based buffers are typically added as pre-made stock solutions (e.g. 10 ml 0.03% HSA in ddH_2O added in making 1 liter of PAG buffer).

3. Prepare double-Percoll gradients in each 50–ml polystyrene conical tube by first adding ~12.5ml of the 55% IPS. Carefully insert drawn-out pastuer pipets into each solution so that tips are at the very bottom of each tube. Then, carefully add 61% IPS down through each pipet to initiate underlaying of 55% IPS with an equal volume (12.5ml) of the 61% solution. (**Note**: it may be important to slightly raise and/or twist each pastuer pipet to expel trapped air and thus start the flow of the 61% IPS. To advoid admixing, flow rate of the 61% IPS should not exceed 2 ml/ minute.

An alternative approach in making the gradients is to use 50ml ACCUSPIN tubes (Sigma Chemical Co.). In this instance, 12.5ml of IPS is immediately poured onto the septum pre-inserted in these tubes. The 61% IPS is then forced below the septum by briefly centrifuging the tubes. Upon achieving this, 12.5mL of the 55% IPS is poured directly into each tubes, thus completing formation of the double Percoll gradients. **Note:** After the density centrifugation step, the pre-inserted septum remains at the same interface where basophils accumulate and thus may slightly impede in their retrival.

Alcian Blue and Staining Procedure (adapted from Gilbert and Ornstein, 1975)

1. Prepare saline –EDTA (label as solution A)

0.1 g EDTA in 100 ml normal saline (0.15M)

2. Add in sequence the following to 100 ml dH_20 (label as solution B):

76 mg cetyl pyridinium chloride ($C_{21}H_{38}ClN$)

736 mg lanthanum chloride ($LaCl_3$ 7H_20)

900 mg NaCl

143 mg Alcian Blue

210 µl Teen-20

- Stir for several hours, heating to 65°C if necessary to get into solution (covered)

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- Aliquot (10 ml) and freeze at −20°C.

3. 1N HCL (label as solution C)

Add in sequence the following to a 0.5 ml polypropylene tube:

- 0.025 ml solution A
- 0.025 ml solution B
- 0.0125 ml of the cell suspension to be counted
- 0.0025 ml solution C

Mix well and then add ~0.025 ml of this to a hemacytometer (recommend a Spiers-Levy)

Basophils appear as blue-stained cells.

COMMENTARY

Background Information—By virtue of their capacity to release three categories of mediators hallmark in allergic disease (i.e. histamine, leukotriene C4, and cytokines –IL-4 & IL-13), basophils have become popular indicator cells to monitor the allergic status before and after therapeutic intervention (Frischmeyer-Guerreio and Schroeder 2012). They can also be activated *in vitro* to induced expression of activation markers (e.g. CD63 and CD203c) that are widely used in flow-based assays as surrogate indicators of degranulation and/or priming that correlate with one's allergic status (McGowan and Saini, 2013). In fact, these so-called basophil activation markers (BAT) are often investigated on basophils within whole blood or PBMC, and thus do not require basophil enrichment or purification. Likewise, the fact that basophils are the only cells in the peripheral blood that contain histamine, means their enrichment/purification is also not necessary when investigating functional responses employing this mediator as the readout. However, as evidence mounts demonstrating the role of basophils as modulators (and not simply effectors) of allergic inflammation (Marone, 2014), more sophisticated experiments utilizing pure cell suspensions are required to explore their bology. And, whereas achieving this goal proved quite difficult just ~15 years ago, advances in using immunomagnetic beads have dramatically eliminated many obstacles. Indeed, the technique presented herein, which combines density centrifugation enrichment with negative selection, has made it possible to routinely isolate basophils to purities exceeding >99%. It is worth noting that cell-sorting protocols have also been utilized in purifying basoohils, especially in isolating mouse basophils. While seemingly desirable because of relative ease, we find these to be undesirable, as many basophils appear to degranulate during the procedure. Moreover, prolonged machine time is often necessary in order to purify relatively large numbers of basophils, given their rarity in PBMC. In contrast, we have long used the protocol herein to purify basophils from whole blood but also from leukopacks generated as by-products during hemapheresis (i.e. TRIMA cassettes) (Schroeder and Sobotka, 2001).

Critical Parameters and Troubleshooting—The percentage of basophils obtained can vary considerably between donors. In addition, the degree of basophil enrichment following the double Percoll gradient centrifugation can also vary among subjects and typically ranges

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between 5–15%. A higher degree of basophil enrichment is often achieved by selecting donors who have greater numbers of basophils, but this is not always the case. Basophil percentages 25%, while rare, are possible with select subjects.

The performance of the density centrifugation step is most sensitive to variations in the 10× PIPES buffer. For this reason, we do not recommend deviations in making this buffer. Otherwise, Percoll gradients must be carefully constructed to avoid mixing the layers. After centrifugation, the layers must be removed with care to minimize mixing of cell populations. Both the BDC and BEC interfaces should be removed as soon as centrifugation is finished in order to prevent prolong exposure to the Percoll. In addition, the speed and temperature of centrifugation are critical and should be set as indicated. A slow start-up centrifuge is necessary to prevent admixing of the Percoll densities. This feature, however, is not readily possible with many modern centrifuges, despite those having programmable acceleration/ deacceleration capabilities. It may be necessary to manually control these steps when possible.

Venous blood drawn into EDTA leads to better recovery than using heprinized blood. Blood should not be held at room temperature longer than required as it may result in more basophils localizing to the BDC interface after density centrifugation.

Anticipated Results—As noted above, the BDC interface, which consists mostly of monocytes and lymphocytes, is similar to PBMC typically isolated using single gradient centrifugation (e.g. Ficoll). On average, the BDC interface comprises 75–80% of the total cells recovered for both interfaces. Upon washing away platlets using four low-speed washes (~150 × g for 10 min.), many other cell types are readily isolated from this fraction, including monocytes, T & B lymphocytes, and immature dendritic cells using a variety of positive and/or negative selection protocols.

Ideally, few basophils (~10%) should localize to the BDC interface following density centrifugation, based on the total number recovered from the two interfaces. If this number routinely exceeds 25%, then hypotonicity should be suspected during troubleshooting and is most likely to occur with the 10× PIPES stock. In contrast, if the total number of cells localizing to the BEC interface exceeds 35%, then one should suspect buffers for hypertonic conditions.

While basophil yields following density centrifugation vary slightly among donors, they generally do not fall below 75% and most often exceed 80–90%. The additional step of purifying basophils using negative selection typically lowers the overall yield to between 50–80%, with purities exceeding >99%.

Time Considerations—The enrichment procedure takes 2h to complete, including preparing gradients and centrifuging and washing of the BEC suspension. To purify basophil using negative selection, another minimum of ~2h is required. Preparation times are kept to a minimum by having all reagents/buffers prepared ahead of getting the blood specimen. Gradient preparation takes ~15 min and should not be hurried.

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