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High-density Ficoll-Hypaque as an alternative method of eosinophil purification

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Abstract: A new method for normodense eosiniphil purification is proposed. It is based on the use of high-density Ficoll-Hypaque (density = 1.114 g mL^{-1}) instead of dextran sedimentation plus standard Ficoll-Hypaque, before discontinuous Percoll gradient centrifugation. Eosinophils from 11 controls and 11 eosinophilic patients were studied. The density distribution of eosinophils was similar in both groups. However, eosinophilic patients showed a significant increase in the absolute count of hypodense eosinophils. Normodense eosinophil purity and recovery were significantly higher in controls as compared with eosinophilic patients ($p < 0.05$). High-density Ficoll-Hypaque allowed separation of mononuclear and polymorphonuclear cells with high purity and recovery using a single centrifugation step. It also reduces one step in eosinophil purification as compared to the standard method. Thirdly, contrary to the single anti-CD16 magnetic bead separation method, it allows the isolation of normodense and hypodense eosinophils. In summary, this method is economic, reliable and permits the purification of different leucocyte populations.

Introduction: Present methods for the purification of human eosinophils from peripheral blood have several limitations [1, 2]. These are due to the small proportion of these cells in the peripheral blood of healthy donors, the different types of eosinophils (normodense and hypodense) and the overlap in the range of densities with neutrophils.

Commonly, sedimentation of red cells with dextran, which results in a leucocyte-rich plasma, followed by Ficoll-Hypaque and discontinuous Percoll gradient centrifugation, is sued as an eosinophil purification method [1]. The major drawback of this protocol is the low yield of pure eosinophils. In order to overcome these difficulties, Hansel *et al.* [3] developed a method using anti-CD160-coated magnetic beads was developed, but this does not allow differentiation between hypodense and normodense eosinophils. Studies comparing normodense and hypodense eosinophils from patients with eosinophilia and healthy donors are thus limited.

A granulocyte isolation technique is reported here using a high-density Ficoll-Hypaque (d = 1.114 g mL^{-1}) [4]. The aim of the study was to evaluate the technique as an alternative method for eosinophil purification. The isolated cells were analysed in terms of cell distribution, viability, purity and recovery.

Materials and methods: EDTA anticoagulated blood samples were obtained from 11 controls and 11 eosinophilic patients, at the Central University Hospital and the Clinical Unit of the Institute of Immunology of the Central University of Venezuela, Caracas. The control group consisted of healthy non-eosinophilic subjects (< 350 eosinophils/mL). The patient group included non-treated eosinophilic patients (490 – 1575 eosinophils/mL) with different aetiologies (mainly allergic or parasitic disease, but not viral infections or chronic diseases).

Whole blood (8 mL) was layered on 5 mL Ficoll-Hypaque (d = 1.114 g mL^{-1}) and centrifuged at 600 *g* for 30 min [4]. Mononuclear and granulocyte layers were removed and washed with PBS-gel (0.01 M phosphate buffer, 2 mM EDTA, 5 mM glucose and 0.1% gelatin) 450 *g* for 10 min at 4°C.

The viability of both well types was > 97% assessed by trypan blue exclusion. Their purity was determined by differential cell count (DCC) in a haematological counter (Coulter, MD Series) and in preparations stained with eosin/methylene blue solution in methanol (analytical grade, Merck) [2]. The yield (percentage) was calculated by dividing the number of each leucocyte subpopulation recovered to the total count of the same subpopulation in peripheral blood per 100.

Afterwards, granulocytes were adjusted to 25–30 × 10^6/mL, resuspended in 1 mL Percoll solution (density 1.070 g mL^{-1}), layered over a discontinuous isotonic Percoll gradient (1.070, 1.080, 1.085, 1090 and 1.100 g mL^{-1}) and centrifuged at 1,600 *g* for 30 min [1,5]. Cells obtained from each Percoll band were removed and washed once with PBS-gel. Then, contaminant erytrocytes were lysed with buffered NH_4Cl solution (150 mM NH_4Cl, 10 mM $NaHCO_3$ and 1 mM EDTA) by mixing it 7 min at room temperature. The cell suspensions obtained were centrifuged at 450 *g* for 10 min at 4°C, and washed twice with PBS-gel (viability > 97%).

The percentage of total eosinophils recovered in each Percoll band was analysed by DCC calculated according to the formula:

$$\text{DCC (\%)} = \frac{\text{Eosinophil number} \times 100}{\text{Total leucocytes per band}}$$

The absolute number of eosinophils per band is as follows:

$$\text{Absolute number} (\times 10^6) = \text{DCC} \times \text{total leucocyte count in each band}$$

The purity of normodense eosinophils preparations (1.090 and 1.100 g mL^{-1} Percoll densities) was performed by DCC and CD16 expression, and confirmed by the presence of IL-5Rα. CD16 and IL-5Rα expression was studied by single direct and indirect immunofluorescence respectively. Cells were incubated with either anti-CD16-FITC or anti-IL-5Rα antibodies plus goat anti-mouse IgG-FITC (GAM-FITC) (Becton Dickinson Immunocytometry Systems, Mountain View, USA).

Table 1. Purity and yield of mononuclear (MN), polymorphonuclear (PMN) and normodense eosinophil (Eo) preparations obtained from 11 controls and 11 patients (means ± SD).

Leucocyte subpopulation	Purity (%)				Yield (%)		
	MN	PMN	Eo		MN	PMN	Eo
			DCC	CD16+			
Patients	88.6 ± 5.2	92.6 ± 4.3	74.9 ± 5.4	30.6 ± 20.0	70.1 ± 15.4	56.2 ± 13.9	9.8 ± 9.3
Controls	92.7 ± 5.1	93.1 ± 5.2	87.6 ± 5.7*	6.2 ± 3.4**	59.5 ± 17	52.7 ± 17.5	25.6 ± 14*

As compared with patients, *$p < 0.05$, **$p < 0.01$.

Flow cytometric analysis was performed in an Epics Profile II (Coulter Electronics, Hialeath, FL, USA), using 488 nm excitation with a green photomultiplier to quantify fluorescence emission, after previous alignment with DNA check fluorescent beads. Mouse IgG1 FITC and GAM-FITC (Coulter Immunology, Hialeah, Miami, Fl, USA) were used as isotype controls.

Results are presented as the means ± SD. Statistical analysis was performed using Student's *t*-test for unpaired data. The limit for significance was taken as $p < 0.05$.

Results: In a single purification step (Ficoll-Hypaque d = 1.114 g mL^{-1}), mononuclear and polymorphonuclear cells were isolated wit high purity and yield from patients' and controls' peripheral blood (Table 1). On the other hand, the purity of normodense eosinophil preparations (Table 1) obtained from 1.090 to 1.100 g mL^{-1} Percoll gradients from patients and controls showed significant differences. Purity was: 74.9 ± 5.5 for patients *vs* 87.6 ± 5.7 for controls ($p < 0.05$) assessed by DCC and 30.6 ± 20.0 for patients *vs* 6.2 ± 3.4 for controls ($p < 0.01$) assessed by CD16 expression.

A high level of IL-5Rα expression (80.2% ± 3.2 for both groups) was also detected in these cell preparations (Figure 1). There were also significant differences ($p < 0.05$) when the yield of normodense eosinophils isolated from patients and controls was compared.

Table 2 illustrates the density distribution of eosinophils in the discontinuous Percoll gradient. Eosinophils from controls and patients showed a similar pattern of distribution. In both cases, the highest percentage of eosinophils was obtained at densities of 1.090 and 1.100 g mL^{-1} (normodense eosinophils).

However, the percentage of normodense eosinophils from controls was higher ($p > 0.05$) as compared with patients. On the other hand, analysis of the absolute count of eosinophils through the discontinuous gradient showed that eosinophilic patients had a significant increase on hypodense eosinophils obtained at density of 1.085 g mL^{-1} as compared with controls ($p < 0.01$).

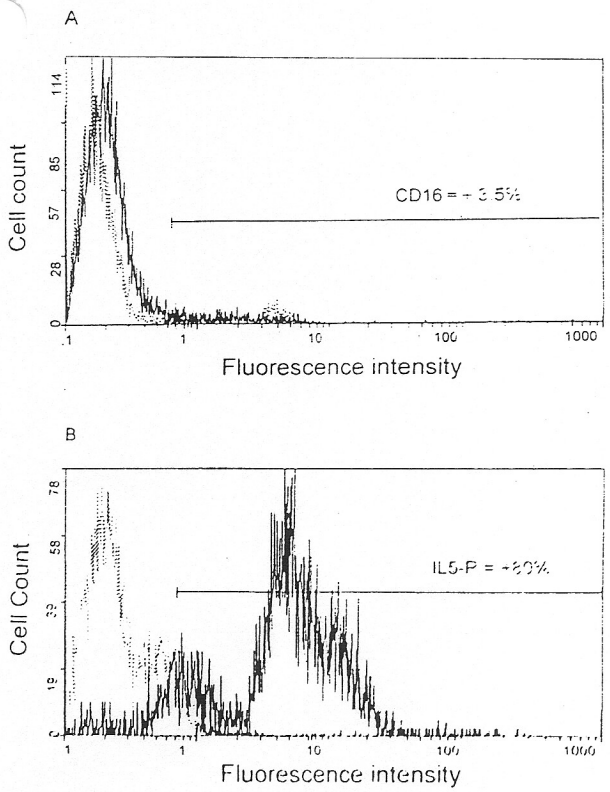

Figure 1. Typical immunophenotype of an eosinophil-enriched cell population labelled with (A) anti-CD16 and (B) anti IL5Rα antibodies. The percentage of positive cells (cursor ( ) was determined by subtracting the isotype control from the two parameters measured.

Discussion: Generally, several laborious steps are required to achieve an acceptably pure eosinophil preparation and the yields can be low. The present study was undertaken to evaluate a high-density Ficoll-Hypaque separation technique prior to Percoll gradient centrifugation, as an alternative method for harvesting normodense eosinophils.

In concordance with previous reports [6], eosinophils isolated from peripheral blood of controls and eosinophilic patients showed similar density distributions. However, as described by Carlson *et al.* [7], eosinophilic patients showed a significant increase on the absolute count of hypodense eosinophils.

Our results show that a differential cell count and CD16 expression represented two efficient parameters for the study

Table 2. Distribution of eosinophils on Percoll discontinuous gradient obtained from 11 controls and 11 patients (means ± SD) of the percentage (%) and absolute (× 10^6) count of eosinophils obtained in each density gradient).

Percoll density (g mL^{-1})	DCC (%)		Absolute count (× 10^6)	
	Patients	Controls	Patients	Controls
1.070	0.5 ± 0.3	0.1 ± 0.1	0.04 ± 0.07	0.1 ± 0.3
1.080	29. ± 0.7	3.6 ± 1.3	1.06 ± 0.5	0.25 ± 0.4
1.085	20.8 ± 6.2	32.8 ± 11.0	6.02 ± 3.5**	1.49 ± 0.8
1.090–1.100	74.9 ± 5.4	87.6 ± 5.7*	1.4 ± 0.9	1.36 ± 1.8

As compared with patients, *$p < 0.05$ *in DCC.*
As compared with controls, **$p < 0.01$ *in absolute count.*

of normodense eosinophil purity. The high levels of IL-5Rα expression confirm these findings.

High density Ficoll-Hypaque has several advantages as compared to conventional methods of eosinophil separation [1]. Firstly, it allowed the isolation of mononuclear and polymorphonuclear cells of high purity and good recovery in a single centrifugation step. Secondly, it saves time and reagents since just two steps of centrifugation are required for the complete normodense eosinophil purification. Thirdly, in contrast to a single anti-CD16 magnetic bead separation [3], the present procedure allows isolation of normodense and hypodense eosinophils.

In conclusion, we present a low cost and less time consuming method for the separation and study of different leucocyte populations. We believe it is particularly useful for the isolation of normodense eosinophils with relatively high purity and recovery.

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