CHARACTERIZATION OF THE BIOPHYSICAL PROPERTIES OF HUMAN ERYTHROBLASTS AS A PRELIMINARY STEP TO THE ISOLATION OF FETAL ERYTHROBLASTS FROM MATERNAL PERIPHERAL BLOOD FOR NON INVASIVE PRENATAL GENETIC INVESTIGATION

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ABSTRACT

Background and Objective. Fetal erythroblasts in maternal circulation represent a valuable source of fetal cell material which can be obtained with non-invasive procedures that do not endanger the fetus. Physical separation techniques have been invaluable in the isolation and characterization of different cells. There are basically two principles that have been used most successfully: separation according to density and separation according to size. In order to determine whether physical separation procedures are capable of purifying human erythroblasts, the biophysical characteristics of these cells were determined.

Methods. Bone marrow particles were obtained from normal adults and peripheral blood buffy coats from blood banks. A single cell suspension was initially fractionated by buoyant density gradient centrifugation. Fractions enriched in erythroblasts were pooled and further processed by velocity sedimentation in order to take advantage of the differences in size of erythroblasts and other cells.

Results. Density distribution curves were drawn after density gradient centrifugation for the different cell types present in the starting cell samples. Separation of the erythroblast-enriched density fractions by velocity sedimentation was successful and a highly purified population of erythroblasts was obtained. Cell size distribution of the different cell types was determined.

Interpretation and Conclusions. This initial study defines the biophysical properties (size and density) of human erythroblasts in bone marrow and peripheral blood and is a necessary preliminary step in setting up the optimal procedure for the isolation of fetal erythroblasts from maternal peripheral blood in sufficient amounts and purity for prenatal non-invasive genetic investigation.
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Key words: erythroblasts, velocity sedimentation, buoyant density gradient centrifugation

The mature red cell is only the final phase of a complex but orderly series of events that are initiated at the time a multipotent stem cell becomes committed to express the erythroid program.[1-3] An understanding of the properties of the cells in the various erythroid compartments and their complex interactions is essential for understanding the physiology of erythropoiesis and for obtaining significant insights into the pathogenesis of disorders affecting the red cell lineage.

The complexity of the cell population in bone marrow has limited cellular and biochemical studies on the differentiation of erythroid and myeloid cells lines; however, cell separation procedures are now available that make it possible to recover different cell types from bone marrow, peripheral blood and single-cell suspensions from solid tissues.[4] A long-sought goal of medical genetics has been the development of prenatal diagnostic procedures that do not endanger the fetus. Data generated in many laboratories worldwide led to the conclusion that fetal cells are undeniably present in maternal blood. Through the last several decades considerable effort has been expended in order to gain access to fetal cells from maternal blood because this would prepare the ground for non-invasive prenatal diagnosis with virtually no risk to mother or fetus. Yet, because the number of fetal cells in maternal circulation is extremely low, no conclusive progress has been made in this field of research in spite of decades of work.[5]

Nucleated red blood cells (NRBCs) are by far the most encouraging candidate for enrichment since they constitute a significant proportion of the red blood cells in fetal blood, but they are very rare in peripheral adult blood. This lineage is mononucle-

ated and has a relatively short life span. Thus these cells possess a full genetic complement. Modern estimates of the ratio of nucleated fetal to maternal cells have been based primarily on DNA analysis for the Y chromosome using either polymerase chain reaction (PCR) or fluorescent *in situ* hybridization (FISH). These estimates range from 1 in 5×10^5 to 1 in 1×10^6. Although an absolute number has yet to be determined, most workers agree on a nucleated cell ratio of about one fetal cell per 10^5 maternal cells, which makes their isolation difficult but not impossible. A prenatal test utilizing fetal cells from maternal circulation would be conditioned by the necessity of recovering as many fetal cells as possible, while eliminating as many maternal cells as possible. The biophysical characteristics (size and density) of a variety of blood and marrow cells are available in the literature, but there is controversy regarding the biophysical properties of NRBCs.[6]

Advances in cell separation procedures which we successfully applied to the isolation of human megakaryocytes,[7] human monocytes[8] and Reed-Sternberg cells[9] suggested to us the possibility of isolating human NRBCs from bone marrow and peripheral blood. The following report describes the characterization of density and size distributions of NRBCs from bone marrow and blood bank buffy coats, in order to optimize the separation of these cells for further experimental investigation.

Materials and Methods

Cell samples

The peripheral blood cells used in these experiments were buffy coat cells separated from normal volunteers through apheresis with a Diaeco-Progress cell separator. Thirty mL of peripheral blood were also obtained from pregnant women undergoing amniocentesis. Bone marrow came from donors for marrow transplantation; the aspirate was mixed with 3 mL of Mc Coy's medium containing 100 IU of heparin. Marrow cells were obtained from marrow particles disaggregated into single-cell suspensions using a dissociation chamber previously described in detail.[10] Briefly, marrow particles were suspended in a dissociation chamber consisting of a leucite reactor with a water jacket. The reactor has two inlet ports at the bottom and a spill over port on the top. The flow rate through the dissociation chamber is controlled by a peristaltic pump set at 25 mL/h. The concentrations of crude collagenase, papain and deoxyribonuclease (Dnase) (Boehringer, Mannheim, GER) in HBSS were 2 mg/mL, 20 µ/mL, and 50 µg/mL, respectively. An upward fluid movement is obtained by the special design of the bottom part of the reactor and the use of a U-shaped magnetic stir bar (Bel-Art Products, Pequannock, NJ, USA). As soon as cells are freed from the marrow particles, they are forced by the upward velocity component through a 150-µm polyester monofilament screen (placed across the top of the reactor) and into a delivery vessel containing Roswell Park Memorial Institute (RPMI) 1640 medium and 10% fetal calf serum (FCS) (Gibco, Breda, The Netherlands). Cells are then washed to minimize contact time with the dissociating enzymes.

Quantitation of cells in suspension

The quantitation of cells was performed at different steps in the purification procedure by optically enumerating nucleated cells in a hemocytometer (improved Neubauer chamber). NRBCs and other cells were counted at a magnification of $320 \times$ using phase-contrast optics. NRBCs were easily distinguished from lymphocytes and monocytes by their large size, a perfectly round nucleus and a homogeneous pattern of chromatin. Paranuclear granules are typical hallmarks of proerythroblasts in phase contrast microscopy. A differential count was made on cytocentrifuge preparations stained with May-Grünwald Giemsa (MGG). At least 200 cells were counted on each smear.

Cell viability was assessed by the trypan blue dye exclusion test. Cell diameters were determined with a calibrated optical micrometer on glutaraldehyde-fixed cells in wet mount preparations by phase contrast microscopy at $1000 \times$. In addition, an electric impedance flow cytometer was used on freshly obtained cell suspensions. Fifty cells were measured and mean size$\pm$SD was calculated for each cell type.

An acid elution technique for hemoglobin F-containing cells was studied in cytocentrifuge preparations stained according to the recommended procedure.[11] Cord blood NRBCs were used as positive control and NRBCs from adult bone marrow as negative control.

Peripheral blood nucleated cells (PBNC) and bone marrow nucleated cells (BMNC) diluted 1:1 with RPMI 1640 were first isolated by density centrifugation on Histopaque-1.119 (Sigma Diagnostic). Cells were washed twice in their own supernatant, made up of their own plasma diluted 1/1 with RPMI, in view of the protection afforded blood cells by plasma proteins. To reduce the platelet load, centrifugation was performed during washing at $250 \times g$ for 8 min. The best separation of platelets from PBNC or BMNC depends on the density of the solution according to the Hawksley-Vand theory. The reader is referred to the paper by van Wie for theoretical details.[12]

Separation of cells by isopyknic gradient centrifugation

Percoll stock solution (SS) (Pharmacia, Biotech) was prepared by mixing 90 mL of commercial Percoll with 8.96 mL of $10 \times$ HBSS without Ca^{++} or Mg^{++}, 0.45 mL (1N) HCl, and 1 mL Hepes buffer (1 mol/L). The required density of Percoll was prepared according to the following formula: Percoll density (g/mL) = (% Percoll SS$\times$0.001186) + 1.0041, where 1.0041 is the density of the saline medium. The final density of the Percoll solutions was adjusted by measuring with a densitometer (K. Paar, AG, Graz, Austria). All solutions used for isopyknic centrifugation were adjusted to 300 mOsm with 4 mol/L NaCl or distilled water, and to pH 7.3 with 1N HCl or 1N NaOH. The osmolality of the solutions was determined with the freezing-point depression technique. Continuous density gradients in the region 1.040-1.119 g/mL were prepared by mixing a Percoll solution of the desired density with a gradient mixer in nitrocellulose tubes. Approximately 200×10^6 cells were resuspended in HBSS containing 0.05% polyethylene oxide (PEO) (British Drug House, Pool, England) before being applied on the density gradient, and carefully loaded on top of each tube and centrifuged for 45 min at $800 \times g$ at room temperature. Following centrifugation, 1 mL fractions were collected from the bottom of the gradient tube. Density marker beads (Pharmacia, Biotech) were run in parallel to check the density gradient. Cells from each fraction were counted for their number and size distributions with a Coulter Counter. Cell density distributions were measured and calculated from the refractive index of each fraction.

Separation of cells by velocity sedimentation

We made use of a velocity sedimentation chamber previously described in detail.[6] Briefly, the separation chamber is filled from the bottom with a linear density gradient generated through a three-channel peristaltic pump (P-3, Pharmacia). The gradient is underlayered with a dense liquid that is immiscible with water (Fluorinert, 3M Co, St. Paul, MN, USA) which brings the gradient up to the end of the capillary tube. Cell suspensions are most stable under conditions of zero van der Waals attractions,[13,14] which may be achieved by adding 12% (v/v) DMSO. This was done to both the cell sample and the gradient. Next, the cell sample is introduced into the upper chamber and the peristaltic pump is reversed, thus lowering the band of cells to the cylindrical part of the device. Before layering the cell suspension onto the density gradient, the cells are counted in a hemocytometer to ensure that their concentration is well below the *streaming limit*, which for this apparatus is 90×10^6 cells. Cell

counting is not performed with an automatic apparatus to avoid having debris or small-sized particles interfere with the electronic counting. This procedure yields a very thin band (less than 1 mm) of undisturbed cell suspension on the gradient. Cells are allowed to sediment at unity gravity for three hours.

Results

Cell yield after bone marrow disaggregation

Dispersion of bone marrow particles with collagenase and/or papain yielded an average of 6.5 (range 2.4-8.5)$\times 10^7$ cells per mg wet weight of marrow particles.

Erythroid and myeloid cells were present at all different stages of differentiatiation. Viability was consistently higher than 90%, as measured by the trypan blue exclusion test.

Peripheral blood nucleated cells lighter than 1.119 g/mL, isolated from blood bank buffy coats averaged 1.4 (range 0.9-1.7)$\times 10^9$.

Erythroblast concentration after isopyknic centrifugation

The yield of all cells from the gradient after two washings was 65% (range 52-76%). The mean distribution profile of five normal bone marrow samples is shown in Figure 1. All samples have been adjusted to the same starting cell number. The morphologically identifiable cells of the granulocytic and erythroid series increased their densities with maturation. Peak densities were: myeloblasts 1.062, promyelocytes 1.073, myelocytes 1.077, metamyelocytes 1.079, and mature neutrophils 1.086. The distribution of erythroblasts (ER) was complex, but in any event two separate bands of hemoglobin-containing cells were often observed on the gradient. Differentiation within the erythroid series was reflected as an increase in density. Many large proerythroblasts were found in the upper region of the gradient, while many more mature erythroblasts were found in the lower region of the gradient. We did not observe a major difference between the density distribution of erythroblasts from bone marrow and that of those from peripheral blood.

Density gradient centrifugation resulted in a slight enrichment of marrow erythroblasts due to the broad distribution profile of the cells, which largely overlaps the density distribution profiles of myeloblasts, myelocytes and promyelocytes (Table 1). It must be emphasized that these were relatively low resolution gradients, since they covered a wide density range with relatively few fractions collected. If the number of fractions collected were increased, then the absolute number of erythroblasts (in the case of peripheral blood) would be too low to draw reliable curves. Furthermore, we wish to draw attention to the fact that the fractions with the highest cell purity did not coincide with the peak density of the different cells.

The procedure described yielded highly reproducible density gradients as confirmed by density

Table 1. Quantitative results of concentrating ER from bone marrow. Results are the mean values from five normal cases.

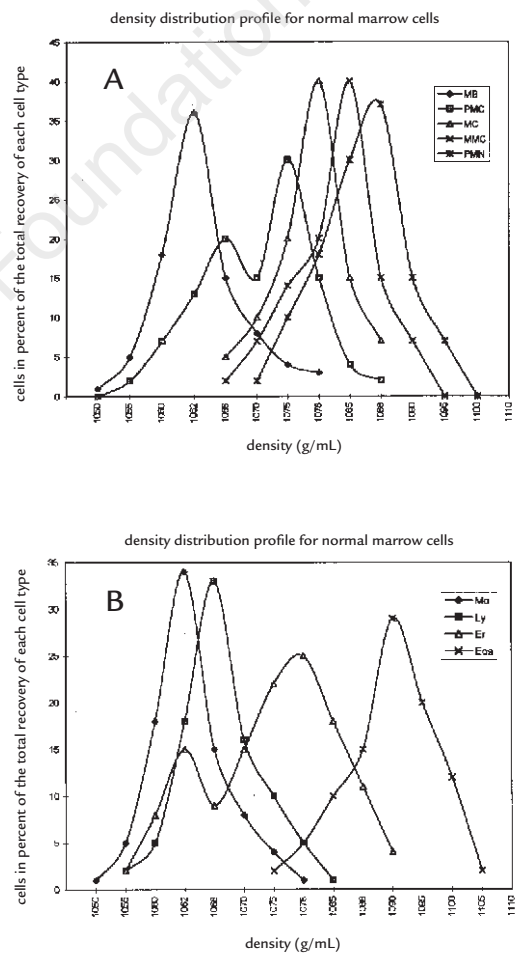| Cell suspension	Yield (total ER±SDx10)	Recovery from previous step (%)	Purity (%)	Viability (%)
Starting cell suspension	28.4±6.7			85
After density centrifugation	12.7±4.3	65	15	80
After velocity sedimentation	10.4±1.1	82	70	78

Figure 1. Buoyant density distribution of marrow and blood cells. a. Density distribution of unfractionated marrow cells. b. Density distribution profiles of several marrow cells. Promyelocytes show a bimodal distribution with two density peaks. Gradient was monitored with density marker beads. *Legend: MB myeloblasts, PMC: promyelocytes, MC: myelocytes, MMC: metamyelocytes, PMN: mature neutrophils, Mo: monocytes, Ly: lymphocytes, ER: erythroblasts, Eos: eosinophils.*

Table 2. Cell diameter and densities of several cell types in peripheral blood.

Cell type	Cell diameter (mean μm±SD)	Cell density (g/mL)	Velocity sedimentation (mm/h)	Recovery (%)
Lymphocytes	9.7±1.4	1.054-1.077	4.9 (6.3-3.0)	87±12
Lymphoblasts	13.0±1.2	1.050-1.073	6.0 (7.8-4.0)	82±9
Erythroblasts I	10.4±1.2	1.065-1.085	5.1 (6.4±3.0)	89±12
Erythroblasts II	14.5±2.2	1.075-1.093	7.4 (9.0-5.2)	80±8
Neutrophils	12.6±1.8	1.084-1.088	n.a.	n.a.
Monocytes	14.4±1.2	1.058-1.064	n.a.	n.a.
Eosinophils	12.4±2.0	1.085-1.098	n.a.	n.a.
Basophils	10.6±1.6	1.072-1.078	n.a.	n.a.

cedure. Figure 3b shows the same cell fraction after the Kleihauer reaction; indeed, while erythrocytes were completely negative, erythroblasts were still positively stained by eosin.

The Kleihauer reaction on isolated erythroid cells was quite surprising. While 3 min of acid elution were sufficient to obtain complete removal of Hb from erythrocytes, a much longer time was needed just to achieve only reduced staining of normal erythroblasts from bone marrow or peripheral blood.

Discussion

The experiments performed during this investigation were designed to determine the physical properties of human NRBCs and other bone marrow and peripheral blood cells as a preliminary step to fetal NRBC isolation from maternal peripheral blood. It is in fact critical to increasing the efficiency of NRBC isolation to identify which marrow or blood cells contaminate the NRBC-containing fractions. While density and volume distribution profiles of marrow NRBCs are available, little is known of the biophysical properties of peripheral blood NRBCs, presumably because of the extremely low number of these cells in circulation, which has prevented reliable curves from being drawn.

Many previous studies have shown that cells of a given volume may be heterogeneous in density and that cells of a given density may have a wide volume distribution. These considerations suggested that successive density-based and size-based separation procedures would frequently be fruitful. Velocity sedimentation at unity gravity, which separates cells according to their size, has been highly developed and many excellent separation trials using this approach have been reported in the literature. The beauty of this method lies in its simplicity.

Cell separation by buoyant density gradient centrifugation (BDGC) is closely related to the previous method. When a heterogeneous cell population is centrifuged in a density gradient whose density at the bottom is greater than the density of the different cells present in the sample, the cells will float to a position in the gradient where the density of the solution is identical to that of the cells, provided the product of time and gravitational field exceeds a certain value. One distinct advantage that contributes to the widespread use of isopyknic gradient sedimentation is that a large number of cells can be fractionated simultaneously. This technique is particularly useful when employed sequentially with other physical separation procedures such as velocity sedimentation. These procedures complement each other since one enriches cells according to their density and the other according to their volume. Therefore instead of choosing between them, they should be used together.

Various methods have been employed to enrich for nucleated red blood cells from peripheral maternal blood. Bianchi et al.[15] initially flow sorted NRBCs using an antibody against the transferrin receptor (CD71). Subsequently, further data suggested that a combination of anti-CD71 with other antibodies, e.g. anti-glycophorin A antibody or anti-CD36 (thrombospondin receptor), would further improve the results.[16] Ganshirt-Ahlert et al.[17] explored the use of a triple-density gradient followed by magnetic activated cell sorting (MACS) to isolate NRBCs, and claimed satisfactory results. Zheng et al.[18] also reported success using MACS to deplete maternal peripheral blood of leukocytes, followed by detection of fetal erythroid cells with an antibody against fetal hemoglobin. Overall, the preliminary data on NRBCs have been encouraging[19] but the proportion of fetal cells in enriched samples is still low, e.g. only 0.001 to 4.8% of a fluorescent activated cell sorter (FACS) enriched sample consisted of fetal cells.

More recently, Bianchi et al.[20] developed an intracellular staining protocol that combines fluorescein isothiocyanate- or phycoerythrin conjugated anti-MoAb with the DNA binding dye Hoechst 33342 to identify and flow sort fetal erythroblasts from maternal blood. They achieved a purity of 4.5-44.5%, but the absolute number of fetal cells sorted was extremely low (7-107). Furthermore, FACS separation is still too expensive and complicated a method of enrichment to be applied routinely.

Surprisingly, fetal cells have been collected primarily by magnetic activated cell sorters and fluorescent activated cell sorters, while physical separation procedures have received little attention, despite the fact that the latter are among the most powerful methods for the purification of homogeneous cell populations and that during the last few decades thousands of reports have described the separation of many different cells with these techniques. Only step gradient centrifugation has been used as a preliminary step to MACS or FACS, resulting in contradictory results. While most investigators use as the starting mononuclear cell frac-

tion cells < 1.077 g/mL, Bhat claims 25-fold enrichment in the cell fraction > 1.077 but < 1.109.[21]

These are two arguments against the use of the step gradient; the principal one is that cells are concentrated at the density step interface, the volume of which is small compared to that of the total gradient. The presence of this high concentration of cells at the interface impedes the redistribution of the cells in the gradient. Indeed a limiting assumption inherent in this technique is that there must be

infinite particle dilution, that is no other particles close enough to affect the sedimentation of the particle in question.

Leif has gone so far as to state that "*unless there is an overriding preparative reason and the system has already been well studied with linear gradient, the results obtained with step gradient are not suitable for publication in reputable journals*".[22]

The very low number of fetal erythroblasts in maternal blood does indeed demand the greatest
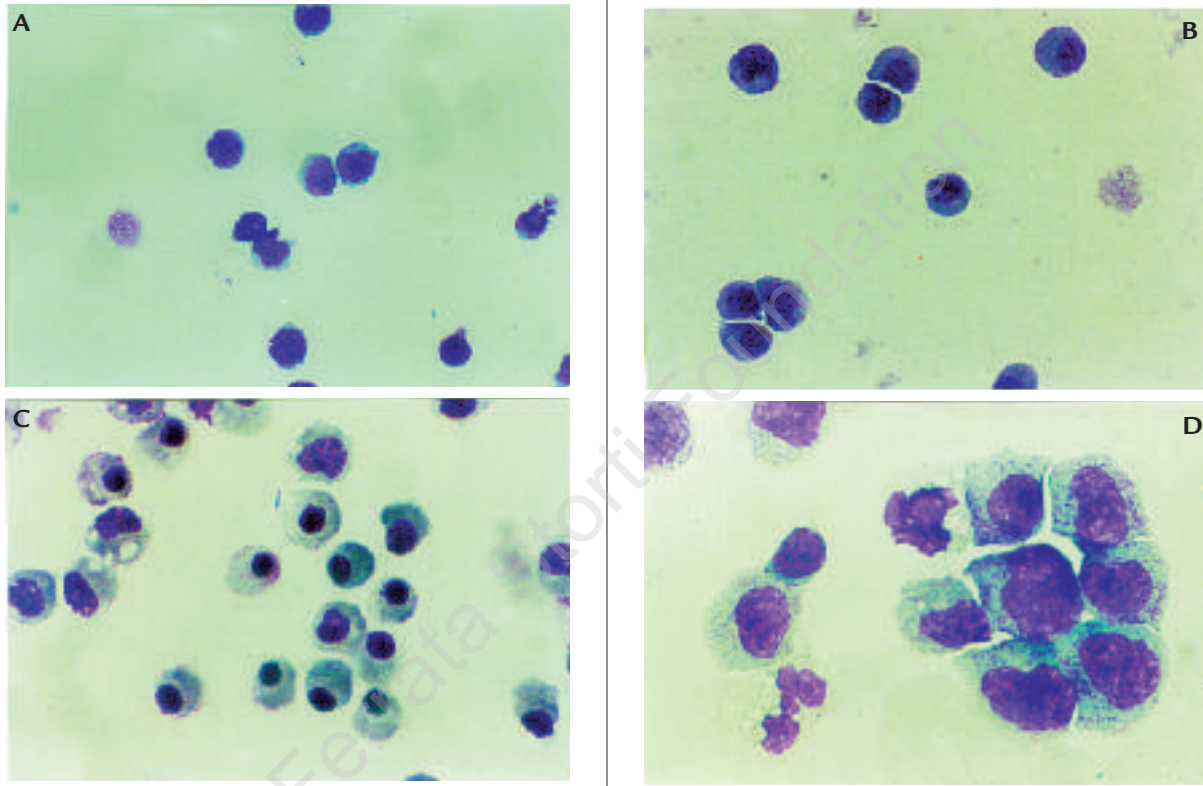

Figure 2. Isolated cells after velocity sedimentation of an erythroblast-enriched cell suspension. a: myeloblasts; b: myelocytes; c: erythroblasts, d: promyelocytes.
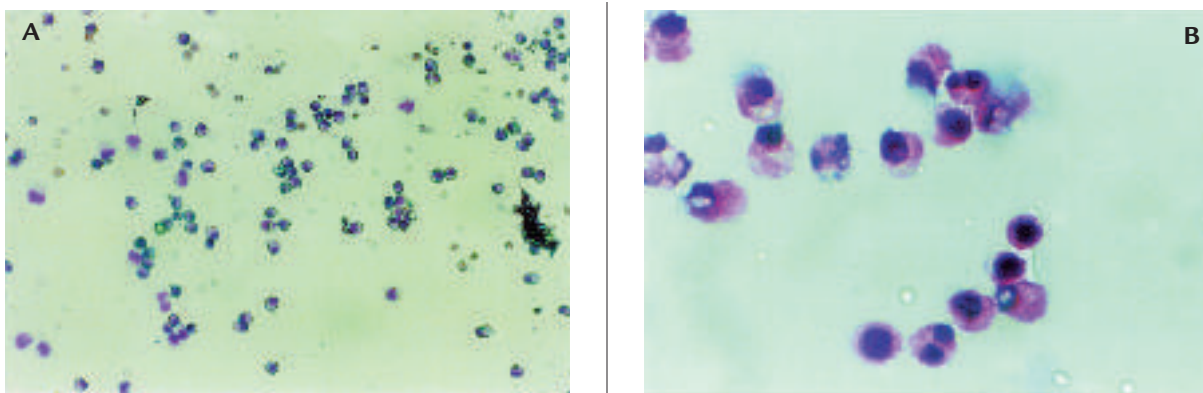

Figure 3. A: erythroblasts from 25 mL of maternal peripheral blood at 12th week of gestation isolated up to homogeneity. Rare blast cells are the only contaminants. B: the same cell fraction after Kleihauer-Behtke reaction. Surprisingly enough NRBCs are still positively stained by eosin even after prolonged acid elution.

accuracy and rigorous attention to detail in order to maximize cell yield. Any loss of fetal cells must be minimized because of the multiplicative effect of each step in the manipulation of the maternal sample. It might be worth pointing out that cell separation by step density gradient centrifugation in a regular tube consistently results in 40±10% cell loss, a figure which might not be relevant when the cells of interest are abundant but one that is quite dramatic when the cells to be isolated are present in minimal amounts.

Our approach is completely new in so far as we employed only physical separation procedures which fulfilled the three major requirements for a routine noninvasive prenatal diagnostic test: that they be rapid, simple and unexpensive.

The gradient separation procedure was highly reproducible and resulted in clearly defined distribution profiles of morphologically identifiable cells. All cells showed homogeneous distributions except for erythroblasts and promyelocytes, which both showed bimodal distributions with two peaks: one in the hypodense and one in the normodense part of the gradient. In general, the rule that younger cells are always less dense than mature ones was followed.

Velocity sedimentation at unity gravity worked well with both PBMC and bone marrow single-cell suspensions. The purification of NRBCs was better in the case of peripheral blood because the major contaminants of the NRBC fraction after buoyant density gradient centrifugation are lymphocytes, whose volume is much smaller than that of NRBCs. By contrast, the fraction containing NRBCs after BDGC of bone marrow cells is contaminated by many myeloid precursors whose volume overlaps that of erythroblasts, thus making complete purification with only physical procedures impossible. An unexpected and unlooked for result was the isolation, in great purity, of other marrow cells: myeloblasts, myelocytes and promyelocytes.

The aim of this paper was not to provide a detailed protocol for the optimum separation of different cells in bone marrow or peripheral blood, but rather to give the necessary biophysical background for devising the most appropriate protocol for fetal erythroblast enrichment or purification.

This study represents a necessary preliminary step toward our goal of isolating fetal erythroblasts from maternal peripheral blood in sufficient amounts and purity for prenatal non-invasive genetic investigation. This next step is presently in progress in our laboratories.

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