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Concentration of Immature and Mature Granulocytes from Normal Human Bone Marrow (38140)

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Although there are numerous reports in the literature describing methods for separating various cell types in bone marrow suspensions (see monograph by Cutts (1)), to our knowledge there are no methods available yet for obtaining mature and immature granulocytes from human bone marrow in sufficient quantity and purity for biochemical studies. Previously we described a Ficoll density centrifugation procedure for obtaining enriched fractions of immature granulocytes from guinea pig bone marrow (2). No enrichment of mature granulocytes was obtained by this method, however. Furthermore, attempts to apply this separation procedure to human bone marrow cell suspensions have been unsuccessful due to differences in the densities of human and guinea pig granulocytes. In the present study we describe several modifications of our previous cell separation method which make it possible to obtain substantially enriched fractions of both immature and mature granulocytes from normal human bone marrow.

Methods. Preparation of Marrow Suspension. Fifteen ml of bone marrow were aspirated from 2 sites in the posterior iliac crest of normal volunteers and immediately diluted, in the absence of anticoagulant, with 135 ml of medium containing 0.15 *M* NaCl, 0.005 *M* KCl and 0.01 *M* sodium phosphate buffer, pH 7.4 (NaKP). The cells were collected by centrifugation at 700*g* for 5 min, washed once with 50 ml NaKP, and diluted to a final volume of 48 ml with NaKP.

The marrow suspension was preferentially freed of erythrocytes and their precursors, using the hypotonic lysis procedure described by Fallon *et al.* (3). After lysis the leukocytes were washed twice in the 50 ml NaKP by centrifugation at 400*g* for 5 min. Prior to the last wash, the cells were filtered through 2 layers of silk filter cloth (average pore size approximately 0.04 mm^2, Joymar Scientific, New York, NY). The cells were diluted to 10.3 ml and a small aliquot was saved for cell counts and smear preparations. This cell suspension served as the starting cells (ST) for the separation procedure described below.

Concentration of Immature and Mature Granulocytes. Initial attempts to isolate immature granulocytes from human marrow suspensions by the Ficoll density centrifugation procedure used previously for guinea pig marrow (2) resulted in heavy contamination of the immature cell fraction by mature cells. This problem was overcome by lowering the concentration of the Ficoll in the centrifugation medium from 17% to 15% (w/v). It was also found more convenient to mix the cells with the Ficoll medium prior to centrifugation rather than to use the more cumbersome procedure of layering the cells over the Ficoll medium which we described previously (2). Briefly, the new procedure consisted of the following steps: 15 ml of ice-cold 25% Ficoll (av. MW, 400,000, Pharmacia, Uppsala, Sweden) in NaKP were mixed thoroughly with 10 ml of cell suspension (containing 2-4 × 10^7 cells per ml) in a 50 ml plastic Falcon centrifuge tube (Bioquest, Cockeysville, MD.). The tube was centrifuged in a refrigerated (4°), International centrifuge, first at 200*g* for 5 min and then at 700*g* for 50 min. The supernatant fraction (FS) containing chiefly immature granulocytes, was carefully removed

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and saved. The cell pellet (FP-1) was processed further for mature granulocytes as described below. The FS fraction was diluted approximately 4-fold with NaKP and bovine serum albumin (Armour Pharmaceutical, Chicago, Ill.) was added in a final concentration of 0.04% to prevent the cells from sticking to the sides of the tube during centrifugation. The cells were collected and washed once with NaKP by centrifugation at 700*g* for 5 min. The final cell pellet was suspended in a suitable volume of NaKP for cell counts and smear preparations.

Mature granulocytes were obtained by processing the FP-1 fraction as follows: the cells were resuspended in 5 ml NaKP and mixed with 20 ml 25% Ficoll (in NaKP). This mixture was then centrifuged at 700*g* for 30 min and the supernatant fraction was discarded. The latter fraction, representing about 40%–50% of the starting cells, had a differential count similar to that of the unfractionated cell suspension. The cell pellet was washed once with 50 ml NaKP containing bovine serum albumin (0.04%) and then was resuspended in NaKP for cell counts and smears. This fraction was designated as FP-2.

Absolute and Differential Cell Counting. Absolute cell counts were performed with a Sanborn-Frommer cell counter (Hewlett Packard, Sanborn Div., Waltham, MA). Smears for differential counting were prepared by means of a cytocentrifuge (Shandon Scientific Co., Sewickley, PA). The cells used for smears were suspended in fetal calf serum (Microbiological Assoc. Inc., Bethesda, MD.) in order to minimize cell breakage during centrifugation in the cytocentrifuge. The staining procedure for differential counts has been described previously (2).

All cells in the polymorphonuclear (PMN), band, and metamyelocyte stages of maturation were classified as mature granulocytes. Cells at the myelocyte, promyelocyte and blast stages were classified as immature granulocytes.

Results and Discussion. The results of differential counts of the cells in four different preparations of starting cells (ST) and fractions obtained by the separation procedure (FS, FP-2) are shown in Table I. The FS

TABLE I. Differential Counts of Bone Marrow Fractions.[a]

		% Mature	% Immature		% Other		
(Fraction)	(Total cells) $\times 10^{-7}$	(PMN + Band + Metamyelocyte)	(Myelocyte)	(Promyelocyte + Blasts)	(Lymphocyte)	(Eosinophil)	(Basophil)
ST	28	69	17	3	7	3	1
	(21–38)	(67–72)	(15–20)	(2–5)	(6–9)	(1–3)	(0–1)
FS	1.7	8	45	31	13	1	2
	(1.1–2.3)	(2–13)	(36–49)	(25–35)	(5–16)	(0–1)	(1–3)
FP-2	8.0	85	9	0	2	4	0
	(6.0–11)	(78–88)	(5–13)	(0–1)	(0-2)	(0–5)	(0)

[a] Results are the averages of four different cell preparations. The range is shown in parenthesis.

fraction contained 76% immature granulocytes, representing a 4-fold enrichment over the starting cells. The predominant cell types in this fraction were myelocytes (45%) and blasts (31%). The FP-2 fraction contained predominantly mature granulocytes (85%). Photomicrographs of FS and FP-2 cell preparations shown in Fig. 1 are typical of the degree of enrichment of immature and mature cells obtained. The cells in both fractions ap-

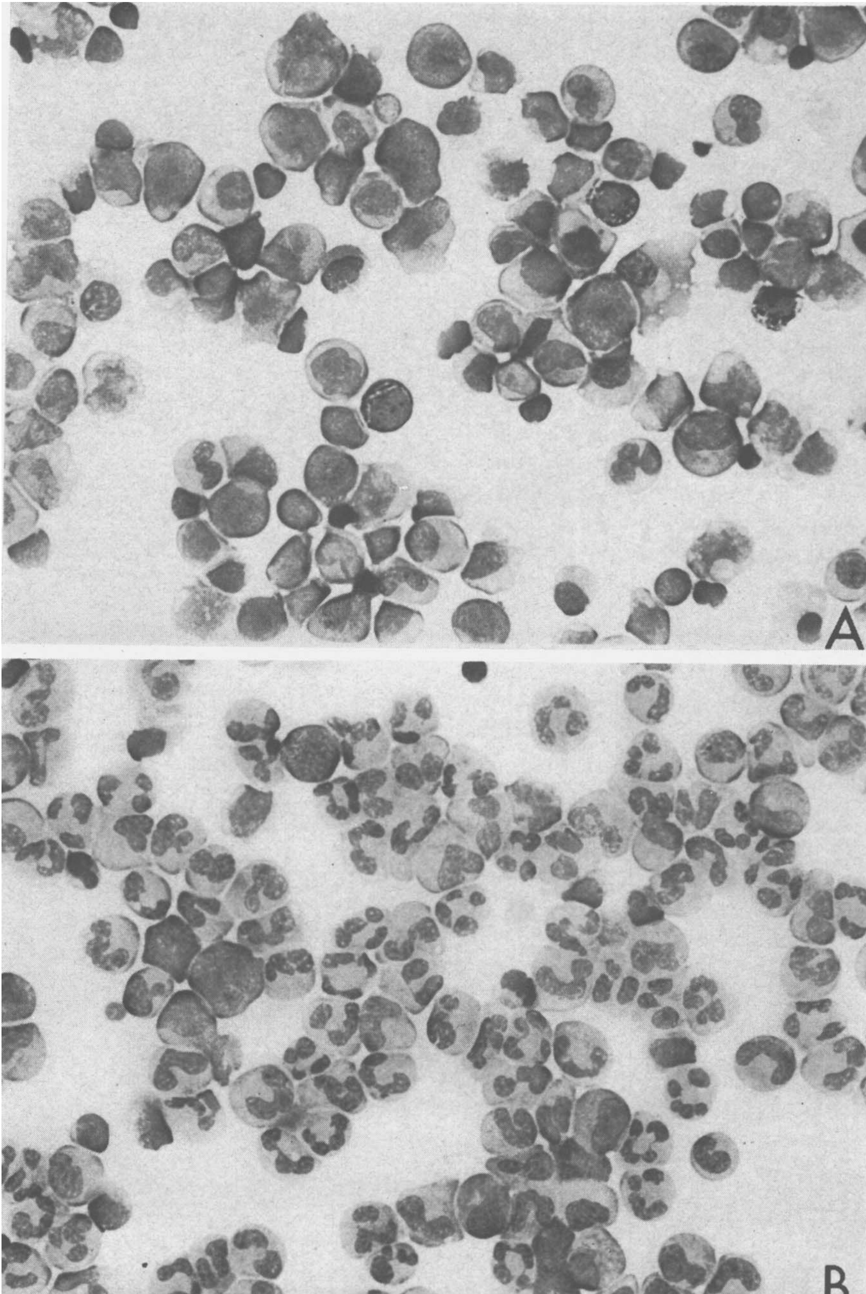

FIG. 1. Photomicrographs of cell fractions isolated from human bone marrow. A. Immature neutrophil granulocytes, (FS). B. Mature neutrophil granulocytes (FP-2). (Magnification, 560×).

pear well preserved morphologically and at least 90% of the cells were found to be viable on the basis of their ability to exclude trypan blue. It has also been shown in previous studies (2) that guinea pig bone marrow cells retain their capacity for DNA synthesis after exposure to separation conditions similar to those used in the present study. It should be noted that no anticoagulants were used in the previous or the present study because we found immature cells treated with heparin or acid-citrate-dextrose to be poorly preserved after the centrifugation procedure.

It is evident from the data in Table I that the high enrichments of immature and mature cells are obtained at the expense of overall recovery of cells (35%). The numbers of immature and mature cells recovered from a single donor, however, were such that significant biochemical comparisons can be made between these cell fractions. For example, we have shown that the mature cell fraction contains about four times as much cytidine deaminase activity as the immature fraction and that immature leukemic cells are similar to normal immature granulocytes with respect to this enzyme activity (4). In future studies we plan to further characterize the biochemical constituents and biosynthetic capacities of these cell preparations.

Summary. Highly enriched fractions of mature and immature granulocytes were isolated from human bone marrow cell suspensions by Ficoll density centrifugation. Fractions of immature cells contained myelocytes (45%), promyelocytes plus blasts (31%), and only 8% mature neutrophils. Fractions of mature cells contained 85% cells at the metamyelocyte to polymorphonuclear stages of maturation and only 9% immature neutrophils. The cells were well preserved morphologically and are useful for biochemical studies of cell maturation.

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