

**Bone Marrow Lymphocytes: Their Isolation, Colony-Forming  
Capacity and Graft-Versus-Host Potential**  
**I. The Isolation and Characterization of Subpopulations of  
Bone Marrow Lymphocytes<sup>1</sup> (35475)**

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(Introduced by L. R. Wasserman)

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Conflicting reports have appeared in the literature in recent years concerning the role of bone marrow lymphocytes as hemopoietic stem cells or immunologic entities (1-6). These issues have not been resolved by studies employing whole bone marrow where the mixed cell populations make certain interpretations difficult. Secondly, no consistently effective techniques have been previously available for isolating relatively pure fractions of the various cell types to study their functional potentiality. Prior attempts to separate bone marrow into its cellular components have utilized the properties of differential surface adherence of cells to fibers (1, 7), or differential sedimentation by size and density (4, 8-10). Some of these methods have provided fractions significantly enriched with lymphocytes, but these fractions also contained large numbers of contaminating cells, such as blasts, whose presence is complicating and renders definitive interpretations impossible. The present study was undertaken to develop a more refined separation procedure that might yield purer fractions of bone marrow lymphocytes, and possibly facilitate the characterization of these lymphocytes by a variety of biochemical and functional properties.

*Materials and Methods. a. Preparation of*

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*bone marrow cells.* Marrow used for each experiment was flushed from the femora of either CF 1 mice or Charles River rats, using Tris-buffered minimum essential medium (Tris-MEM, pH 7.2). After careful aspiration, the cells were filtered through a single layer of sterile Kimwipe (Kimberly-Clark), and concentrated by centrifugation at 1000 rpm for 10 min. The cell button was suspended in a 1:1 solution of fetal calf serum and Tris-MEM. Varying cell numbers were layered on sucrose-serum gradients to determine optimum cell numbers.

*b. Cell separation on sucrose-serum gradients.* Linear density gradients were prepared with a 10-ml gradient maker containing a lighter solution of 5% sucrose in Tris-MEM (diluted to half strength), and 20% fetal calf serum. The heavier component was a 15% sucrose solution containing 20% serum. This procedure, which is a modification of that described by Osmond (9), substituted calf serum for autologous serum and introduced Tris buffer to maintain a physiologic pH. The gradients were prepared fresh in 14-mm diameter cellulose nitrate tubes (Servall). One ml of cell suspension was layered on top of each gradient and the preparation was centrifuged at 1000 rpm for 5 min at 4° in a PR2 International Centrifuge (Head No. 253). The fractions into which the marrow cells separated were collected individually by Pasteur pipette from the top of the gradient. The cell fractions were washed in MEM, counted by hemocytometer, and smeared for microscopic analysis. Cell viability was assessed by trypan blue exclusion. Incorporation of <sup>125</sup>I deoxyuridine (<sup>125</sup>I DU, 0.5 μCi/ml) and triti-

TABLE I. Cell Yield in Absolute Numbers (Abs) and Percentage of Bone Marrow Fractions Separated on Sucrose Density Gradients.

Expt. no.	Marrow donor	Fractions: 1		2		3		4	
		Abs $\times 10^6$	%	Abs $\times 10^6$	%	Abs $\times 10^6$	%	Abs $\times 10^6$	%
5	Mice	5.5	2.4	9.5	4.1	5.0	2.2	210.0	91.3
6		1.2	0.7	7.8	4.4	13.5	7.5	158.5	88.1
7A	Rats	1.8	1.7	4.2	4.0	5.4	5.1	94.2	89.2
7B		1.2	1.1	3.0	2.8	4.2	3.8	102.6	92.3
8		1.2	0.7	7.2	4.2	13.2	7.6	151.2	87.5
9		1.6	1.0	12.0	7.4	4.8	3.0	143.2	88.6
	Mean (%)		1.27		4.48		4.87		89.50
	$\pm$ SEM		0.27		0.63		0.93		0.77

ated thymidine ( $^3\text{HTdr}$ ,  $1 \mu\text{Ci/ml}$ ) into DNA during a 2-hr pulse at  $37^\circ$ , was also used as an index of the metabolic condition of the cells following their removal from the gradient. The cell fractions exposed to  $^3\text{HTdr}$ , were processed by autoradiography and the  $^{125}\text{I}$  DU-treated cells were washed three times and the percentage of the label incorporated was determined by well-type scintillation counter. Differential cell counts were performed on Jenner-Giemsa stained smears by enumerating 1000–4000 cells/fraction.

**Results.** Bone marrow cells obtained from mice or rats sedimented into five fractions on the sucrose-serum gradient. The two lightest fractions containing only a small number of cells, were combined for convenience and together designated Fraction 1. Fraction 2 appeared as a dense, well-defined band which contrasted sharply with the slightly cloudy layers of Fractions 1 (above it) and 3 (below it). A clearly circumscribed button of cells sedimenting to the bottom of the tube was called Fraction 4. Analysis by light microscopy revealed that the three uppermost fractions (Fractions 1, 2, and 3), contained a predominance of lymphocytes (Fig. 1), the highest percentage of which (90–99%) was present in Fraction 2. Although the majority of the lymphocytes appeared relatively homogeneous, being 7 to 9  $\mu$  in diameter, with a heterochromatic nucleus and pale-staining cytoplasm, Fraction 3 contained in addition, a small percentage of lymphocytes, which featured deep cytoplasmic

basophilia and partially developed euchromatic nuclei (Fig. 2). This small group of cells was designated transitional cells, adopting the terminology of Yoffey *et al.* (11) who described similar cells in the bone marrow of guinea pigs. The contaminating cells in the lymphocyte fractions were orthochromatic normoblasts, neutrophils, and less than 1% undifferentiated blast cells. Cells were classified as blasts if they possessed euchromatic nuclei, nucleoli, and basophilic cytoplasm lacking in specific granulation. The purity of the lymphocyte fractions depended upon the numbers of bone marrow cells layered on the gradient,  $150\text{--}200 \times 10^6$  cells being optimal. Fraction 4 generally contained less than 10% small lymphocytes and virtually all of the large cells including young erythroid and myeloid cells, megakaryocytes, and undifferentiated blasts. This fraction also displayed a striking assemblage of mature polymorphonuclear leukocytes, late stages of nucleated erythroid cells and small numbers of plasma and reticulum cells. Mature erythrocytes in the marrow were distributed mainly to Fractions 2 and 4.

Cell recovery in Fraction 1 was about 1% and roughly 5% each for Fractions 2 and 3 (Table I). This recovery in the three lymphocyte-rich fractions represented 30–40% of the total lymphocytes present in marrow samples containing 25–28% lymphocytes. Cell viability was also high as indicated by trypan blue exclusion test which provided viability counts of 92–99%.

When the marrow fractions were compared

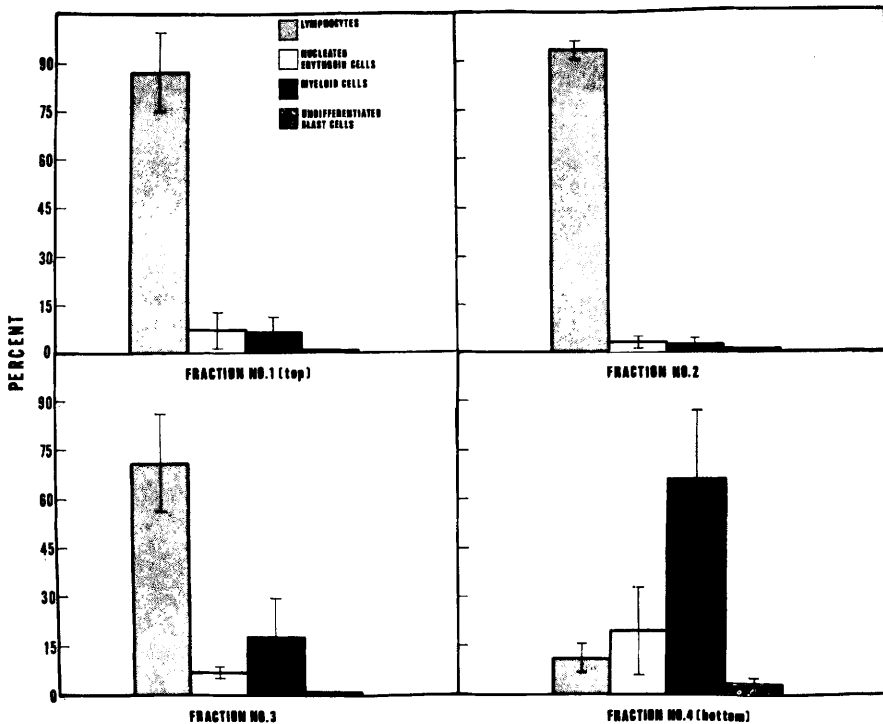


FIG. 1. Differential cell counts of the fractions obtained from bone marrow sedimented through a sucrose-serum gradient. The values represent the mean  $\pm$  1 SD of 7 expts. The fractions are numbered consecutively from the lightest (Fraction 1) to the heaviest (Fraction 4).

for their capacity to incorporate  $^{125}\text{I}$  deoxyuridine into DNA, the data of Fig. 3 were obtained. Each value represents the mean percentage incorporation for quadruple cultures, each containing  $8 \times 10^6$  cells. As shown, DNA synthesis was confined largely to 2 groups of cells, namely, Fractions 3 and 4. Fraction 2, with its almost pure population of small lymphocytes, showed only about 0.5% uptake. The technique thus appeared to have separated one subpopulation of lymphocytes that could be distinguished by its ability to synthesize DNA, from others that were not replicating (Fractions 1 and 2), at least, not during the interval studied. Autoradiographic analysis supported this conclusion. While only an occasional cell was labeled in Fractions 1 and 2, large numbers were found in Fraction 3. The majority of these labeled cells in Fraction 3 were identified as transitional cells (Fig. 2F). A variety of cell types including undifferentiated blasts, and young myeloid and erythroid elements were synthesizing DNA in

Fraction 4 (Fig. 2).

Table II compares the results of some techniques employed by various investigators for the separation of bone marrow lymphocytes. Comparing the sedimentation time, and the purity and cell yield of the lymphocyte fractions, it appears that the present technique and that originally employed by Osmond (9) are the most efficient. The modifications introduced in the present study (calf serum and a Tris-buffered system) provide still greater advantages in purity and cell yield. In addition, the lymphocytes are segregated into different fractions, a feature which facilitates their individual study and possible characterization as subpopulations by a variety of functional criteria.

*Discussion.* The present modification of the original sucrose-serum gradient technique (9) appears to offer a technical advantage in separating lymphocytes from the heterogeneous bone marrow populations. The system obviates the use of autologous serum and

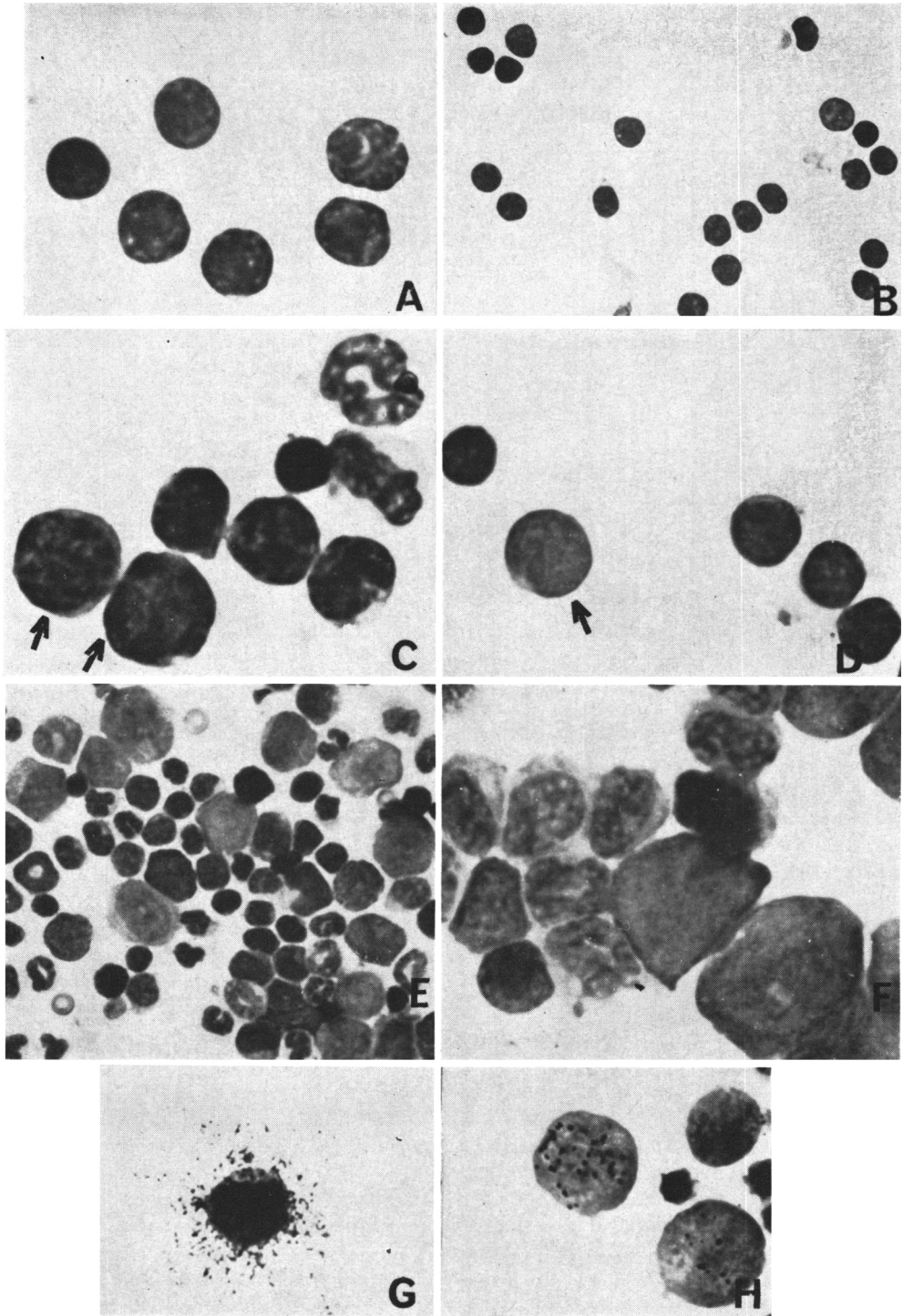


FIG. 2. Photomicrographs of bone marrow cells fractionated on a sucrose-gradient: (A, B) small lymphocytes of Fraction 2; (A) 3880 $\times$ ; (B) 1600 $\times$ ; (C, D) transitional cells (indicated by arrows) of Fraction 3. 3880 $\times$ ; (E, F) cells of Fraction 4. Note the predominance of mature

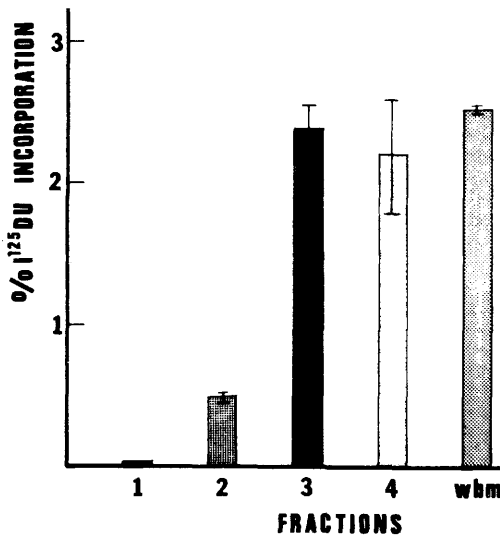


FIG. 3. Percentage incorporation of <sup>125</sup>I deoxyuridine into cell fractions obtained from bone marrow sedimented through the sucrose-serum gradient. The values represent the mean percent  $\pm$  1 SD of quadruple cultures, each containing  $8 \times 10^6$  cells/4-ml cultures, and 0.5  $\mu$ Ci of <sup>125</sup>I DU/ml, in a 2-hr pulse. Fraction 1 had only 2 cultures each with  $0.3 \times 10^6$  cells. WBM: bone marrow unfractionated.

does not require a double sedimentation process to obtain lymphocytes of high purity. The lymphocytes are themselves segregated by size and density into three major fractions which together account for a recovery of 30–40 percent of the total lymphocyte population present in the original marrow sample. This value contrasts strongly with the 5 percent yield reported previously (9).

The technique also avoids some of the undesirable features associated with other systems. Sedimentation of bone marrow cells through an Isopaque gradient requires about 2.5 hours (8). In our experience this procedure results in high mortality, low cell yields, and significant contamination with blastic elements. A time-consuming method employing a double procedure of fiber filtration followed by sedimentation through a dextran gradient (7) achieves increased lymphocyte enrichment, but does not reduce blast cell contamination to acceptable levels. In

contrast, the present technique achieves a striking reduction of blastic elements among the lymphocytes, particularly in Fraction 2. The short sedimentation interval (5 minutes) and careful maintenance of the pH may also have contributed to the high viability and excellent morphologic integrity of the cells removed from the gradient. A 2-hour pulse labeling of the cells with H<sup>3</sup>Tdr or I<sup>125</sup> DU, resulted in levels of incorporation that compared favourably with unfractionated bone marrow, indicating that the cells maintained intact metabolic functions.

Autoradiographic assessment showed that of the three lymphocyte bands, Fraction 3 contained the largest numbers of cells labeled with I<sup>125</sup> DU or H<sup>3</sup>Tdr. These observations corroborate the findings of other reports (11, 12, 13) that lymphocytes in bone marrow synthesize DNA. The present studies further identify this group of cells by physically separating them from a group of non-proliferating lymphocytes. The interrelationship between these subpopulations of lymphocytes remains to be determined. The results of these studies suggest that the present technique may provide a means of classifying bone marrow lymphocytes into subpopulations on the basis of their sedimentation characteristics and proliferative capacity. This would provide a unique opportunity to explore the enigmas of bone marrow lymphocytes in terms of their immunologic and hematopoietic roles. It would also extend previous studies which have indicated the heterogeneity of lymphocytes in terms of their life span (14), migration (15), and immunologic functions (16).

**Summary.** Bone marrow cells sedimented through a linear sucrose gradient containing fetal calf serum, separated into four major fractions. Three of these were lymphocyte-rich fractions that may be as high as 99 percent pure. About 40 percent of total marrow lymphocytes (originally 25–28 percent) were removed in these three fractions. The technique offers the advantage of short sedimentation intervals (5 minutes) and high

granulocytes. Immature cells and few small lymphocytes are also present. (E) 1600 $\times$ ; (F) 3880 $\times$ ; (G) transitional cell of Fraction 3 labeled with <sup>125</sup>I deoxyuridine; (H) blast cells of Fraction 4 labeled with <sup>3</sup>H thymidine; 2910 $\times$ .

TABLE II. A Comparison of Lymphocyte-Enriched Fractions Isolated from Bone Marrow by Various Techniques.

Separation procedure	Marrow source	Separation time	Differential cell counts (%); nucleated cells:					Recovery (%) of original lymphocytes	Ref.
			Lymphocytes	Immature "blasts"	Erythrocytic cells	Myelocytic cells	Other		
Stable-flow free boundary sedimentation	Mice	30 min	61	3.7	NG <sup>a</sup>	0.0	35.3	—	(4)
Unit gravity sedimentation through Isopaque and dextran	Rats and guinea pigs	2-3 hr	70-90	←	→	10-30	→	—	(8)
Surface adherence plus dextran gradient sedimentation	Rats	Filtration time + 30-min sedimentation	86.5	2.0	1.2	8.2	1.8	15.7	(7)
Sucrose autologous serum density gradient	Guinea pigs	10-12 min	Single sedimentation: 72-88 Dual sedimentation: 92-99	NG	NG	NG	NG	5	(9)
Sucrose-calf serum density gradient	Rats, mice	5-min single sedimentation	Fr. 1: 86.3 (70-98) Fr. 2: 94.5 (90-99) Fr. 3: 73.6 (55-91)	0.0 0.05 0.30	7.1 3.0 7.6	6.6 2.4 17.9	0.0 0.05 0.60	30-40	

<sup>a</sup> NG = not given.

reproducibility in the purity and yield of lymphocytes. The technique isolates lymphocytes in a one-step procedure and further subdivides the lymphocytes on the basis of differences in sedimentation and proliferative characteristics. The availability of a technique that may differentiate subpopulations of bone marrow lymphocytes should stimulate future investigations aimed at clarifying the differentiative and functional capacities of these cells.

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