Human Bone Marrow Lymphocytes

I. DISTRIBUTION OF LYMPHOCYTE SUBPOPULATIONS IN THE BONE MARROW OF NORMAL INDIVIDUALS

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ABSTRACT This study was undertaken to determine the proportions and in vitro immune capacities of lymphocyte populations in the bone marrows of normal humans. Relatively pure mononuclear cell suspensions were obtained from bone marrow aspirates by linear sucrose gradient centrifugations. Simultaneous peripheral blood and bone marrow specimens from each individual were assayed for lymphocyte surface markers and mitogen responsiveness. Maximal possible contamination of bone marrow aspirates by peripheral blood was determined by performing aspirates on individuals who had received ^{51}chromium-labeled autologous erythrocytes. Thymus-derived (T) lymphocytes, as determined by the sheep red blood cell (E) rosette assay, comprised 8.6-(±1.6)% of the total bone marrow lymphocyte pool. Bone marrow-derived (B) lymphocytes, as determined by the presence of a complement receptor, made up 15.4-(±1.9)% of the lymphocyte pool whereas 74.6(±2.4)% of mononuclear cells lacked easily detectable surface markers. These findings could not be explained by contamination with peripheral blood lymphocytes since contamination was corrected for in the calculations. Lymphocyte-enriched suspensions of bone marrow cells responded to stimulation with phytohemagglutinin, concanavalin A, and particularly pokeweed mitogen. In vitro incubations of bone marrow and peripheral blood lymphocytes with tritiated thymidine followed by determinations of E and erythrocyte antibody complement (EAC) rosettes were performed. Simultaneous rosette-radioautographs demonstrated that the proliferative potential of bone marrow B lymphocytes was greater than peripheral blood B lymphocytes ($P < 0.01$). On the other hand, the proliferative potential of bone marrow T lymphocytes was the same as that of peripheral blood T lymphocytes. These findings demonstrate that in addition to containing B lymphocytes the normal bone marrow contains a small fraction of T lymphocytes similar to the mature T lymphocyte pool found in the peripheral blood. These T cells most probably enter the bone marrow parenchyma as part of the normal recirculating lymphocyte pool.

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INTRODUCTION

It has been well-established in both animal species and man that the bone marrow (BM)[1] is the source of so-called BM-derived (B) lymphocytes (1). In the postnatal period, the BM serves as the source of stem cells which, under the influence of the thymus, can differentiate into peripheral thymus-derived (T) lymphocytes (1, 2). Several animal studies have demonstrated that in addition to serving as a source of rapidly turning over B lymphocytes throughout life, the BM also contains a small but definite compartment of mature long-lived T lymphocytes (3, 4). These cells are recirculating T lymphocytes which probably do not arise in the BM but enter from the circulation and are part of the larger total body recirculating lymphocyte pool which includes the thoracic duct lymph, spleen, and

[1] *Abbreviations used in this paper:* B, bone marrow derived; BM, bone marrow; C, complement; Con A, concanavalin A; E, erythrocyte; EA, erythrocyte antibody; EAC, erythrocyte antibody complement; FCS, fetal calf serum; HBSS, Hank's balanced salt solution; ^{3}HTdR, tritiated thymidine; MEM-S, Eagle's minimum essential media; PB, peripheral blood; PBS, phosphate-buffered saline; PHA, phytohemagglutinin; PWM, pokeweed mitogen; RBC, red blood cells; SRBC, sheep red blood cells; T, thymus derived.

lymph nodes (5, 6). In addition, mature T lymphocyte functions have been demonstrated in the BM in various animal studies. These include the ability to respond to T-cell mitogens (7, 8), to various antigens (9) including alloantigens (10), and the ability to mediate contact sensitivity (11) as well as the graft versus host reaction (12, 13). On the other hand, there have been conflicting data regarding the presence of a fraction of mature T lymphocytes in human BM. Some studies have found an absence of or minimal T lymphocytes identified by surface markers and response to T cell mitogens in the BM of patients with immunological deficiencies and some normal controls (14–17). Other studies, however, have demonstrated functionally mature T lymphocytes in the BM of children in long-term, chemotherapeutically induced remission from acute lymphoblastic leukemia (18–20). The present study was designed to examine BM aspirates from a large number of normal volunteers for the following purposes: to separate relatively pure populations of BM lymphocytes, to perform comparative studies done simultaneously on the peripheral blood (PB) and BM of each individual, to determine the maximal degree of contamination of BM specimens with PB, to calculate the precise proportions of lymphocyte subpopulations contained within the BM parenchyma, and to characterize the relative proliferative potential of PB and BM lymphocyte subpopulations by simultaneous surface marker and radioautographic studies.

METHODS

Subjects. BM aspirates were done on 37 normal volunteers, 21 men and 16 women, ranging in age from 19 to 25 yr. Details of the procedure were explained to each individual, and informed consent was obtained. The subjects were all in excellent health and were taking no medications at the time of the study.

BM aspirates. The site of the biopsy was the posterior superior iliac crest. The area was prepared with iodine and 75% alcohol and draped. Xylocaine (1%) was used to anesthetize the overlying skin and periosteum. A Rosenthal needle was used and 0.5–1.0 ml of aspirate was withdrawn into a syringe containing acid citrate anticoagulant. A "touch prep" smear was then done and only those specimens which contained macroscopically visible bone spicules and fat globules were used. Only one aspirate had to be rejected throughout the study. Each individual also had a 35-ml sample of heparinized peripheral blood drawn for comparative studies. Total nucleated cell counts were done on the BM aspirate as well as on a sample of PB using a Coulter counter (Model Fn, Coulter Electronics Inc., Fine Particle Group, Hialeah, Fla.). Differential counts (400 cells) on Wright-Giemsa stained smears were also done on both samples by the same observer throughout the study.

Determination of degree of contamination of BM aspirate with PB. Despite the fact that meticulous attention was paid to avoiding gross contamination with PB as much as possible by withdrawing only 0.5–1.0 ml of BM aspirate and accepting only those samples with visible bone spicules, further attempts were made to determine precisely the amount by volume of PB contained in aspirates drawn in this manner. It has been firmly established by radioactive labeling studies that nonnucleated BM erythrocytes (RBC) are intravascular (21). Hence, normal nonnucleated RBC contained in a BM marrow aspirate indicates contamination by RBC from PB contained in BM vascular channels. Eight subjects had BM aspirates obtained while they were partaking in an independent study in which they had been reinfused the previous day with radioactive chromium (^{51}Cr) autologous RBC. Simultaneous with the BM aspirate, a sample of PB was drawn and the radioactivity in equivalent volumes of BM and blood samples was compared to determine the percent contamination by volume of the BM samples with PB. Radioactivity was counted in an automatic gamma counter (Series 1185, Nuclear-Chicago, Des Plaines, Ill.).

Preparation of cell suspensions. BM cells were dispersed from the spicules by repeated aspirations through a 1-ml pipette. RBC were removed from the aspirate by hypotonic lysis. Lymphocyte-rich suspensions were obtained from the BM aspirates by a modification of the sucrose gradient centrifugation method of Osmond (22). Briefly, five sucrose gradients of 15–35% (increments of 5%) in Hank's balanced salt solution (HBSS) and 20% fetal calf serum (FCS) (Industrial Biological Labs., Inc., Rockville, Md.) were prepared. A gradient of 35% through 15% (2 ml of each 5% increment) was layered in a 1.5×9-cm plastic test tube to give a total of 10 ml. BM cells (4×10^7–2×10^8) suspended in 2 ml of Eagle's minimum essential media (MEM-S) (Grand Island Biological Co., Grand Island, N. Y.) were layered over the sucrose gradient and centrifuged at 100 *g* for 7 min at 20°C. The upper 4.5 cm of the gradient containing the lymphocyte-rich suspension was collected, washed three times in MEM-S, and a cell count was performed in a Coulter counter (Coulter Electronics Inc.). A cytocentrifuge smear was made of the suspension and stained with Wright-Giemsa stain. A 400 cell differential count was performed by the same observer throughout the study. The lymphocyte-rich suspension from the upper portion of the sucrose gradient was used for subsequent studies except when indicated.

Mononuclear cells (lymphocytes and monocytes) were obtained from the 35-ml sample of heparinized PB (drawn at the time of the BM aspirate) by the Hypaque-Ficoll method (23).

Proportions of T and B lymphocytes. T lymphocytes from lymphocyte-rich suspensions of BM and PB were identified by their ability to form spontaneous RBC (E) rosettes with sheep red blood cells (SRBC) (24). To 0.25 ml of the BM or PB mononuclear cell suspension (4×10^6 cells/ml) in HBSS was added 0.25 ml of 0.5% SRBC. The mixed cell suspension was incubated at 37°C for 5 min, centrifuged at 100 *g* for 5 min at 4°C, and the supernatant HBSS was removed. 0.5 ml of FCS which had been absorbed twice with SRBC was added, and the pellet was incubated at 4°C overnight. One-half the volume of supernate was then removed and the cells were gently resuspended by shaking. One drop of cell suspension was put on a glass slide and a coverslip applied. 200 lymphocytes were counted by the same observer throughout the study using phase contrast optics at 400× magnification on a Zeiss microscope (Carl Zeiss, Inc., New York). All lymphocytes binding more than three SRBC were considered positive.

B lymphocytes were identified by their ability to bind antibody-complement coated SRBC (25). SRBC coated with rabbit IgM antibody (EA) were a gift from Dr.

Michael M. Frank and were prepared as previously described (26). Fresh mouse serum served as the source of complement (C). 1 ml of mouse serum diluted 1:10 in veronal-buffered saline containing optimal concentrations of calcium and magnesium in 0.1% gelatin was incubated with 1 ml of IgM EA (1×10^8/ml in phosphate-buffered saline [PBS]) for 40 min at 37°C. The resulting EAC were washed three times in 0.01 M EDTA-buffered saline and resuspended in 5 ml of 0.01 M EDTA-buffered saline. To 0.25 ml of the EAC was added 0.25 ml of the BM or PB lymphocyte suspension. The mixture was incubated on a rotator for 40 min at 37°C. A drop of the suspension was placed on a glass slide and counted in the same manner as described above for E rosettes. Monocytes were distinguished from lymphocytes by morphology and by their phagocytosis of latex particles.

In addition to the EAC marker determination for B lymphocytes which was done in all subjects, 10 subjects had, in addition, determination of proportions of B lymphocytes in the BM and PB by Ig surface markers. Relative percentages of B lymphocytes in the BM and PB bearing surface IgG, IgM, and IgA were determined (27) using fluoresceinated goat antihuman IgG, IgM, and IgA antisera (Meloy Laboratories, Inc., Springfield, Va.). The specificity of these antisera was verified by immunoelectrophoresis and by blocking the staining by monospecific nonfluoresceinated anti-Ig. The lymphocyte suspensions were incubated separately with the various fluoresceinated anti-Ig for 45 min at 4°C. The cells were then washed three times with cold PBS, resuspended in glycerol-PBS (9:1), and fluoresceinated cells were counted using a Zeiss fluorescent microscope with a HBO 200 mercury arc, a dichroic reflector 500, a 50 barrier filter, and a FITC 500 excitation filter.

Radioautographic studies of BM and PB. The spontaneous synthesis of DNA by a cell indicates that the cell possesses proliferative capacity or the potential to divide under the proper conditions, since it is felt that DNA is synthesized by cells only in preparation for cell division (28–30). This measurement of spontaneous DNA synthesis has been employed to estimate the life span or state of maturation of cell populations (28–30). To determine the spontaneous DNA synthesis and hence the relative proliferative capacities of T and B lymphocytes in the BM and PB, cell suspensions from eight subjects were incubated with tritiated thymidine (^{3}HTdR) (6.7 Ci/mM, New England Nuclear, Boston, Mass.) at a concentration of 100 μCi/ml of cells for 1 h at 37°C. Because of the difference in volumes of BM aspirates (0.5–1.0 ml) and PB samples (35 ml), the following was done: ^{3}HTdR was added to the unfractionated BM aspirate and incubated as described above. After the incubation, the cells were washed three times with MEM-S, and a smear of the unfractionated aspirate was made. The cells were then applied to a sucrose gradient as described above. E and erythrocyte antibody complement (EAC) rosettes were made separately from the lymphocyte-rich suspension, and cytocentrifuge smears were made of these rosetted lymphocytes which had previously been incubated with ^{3}HTdR. For the PB sample, a 2-ml quantity of blood was incubated for 1 h with ^{3}HTdR as described above. The sample was then washed three times with MEM-S and smears made. For the simultaneous rosette-radioautograph studies on PB, Hypaque-Ficoll separation was first performed on 30-ml blood samples. The mononuclear layer was incubated with ^{3}HTdR for 1 h, as described above, and E and EAC rosettes were prepared similar to the BM samples. The smears for radioautography were coated with Kodak NBT-2 emulsion (Eastman Kodak Co., Rochester, N. Y.), exposed in the dark at 4°C for 7 days, developed with Kodak D-19 (1:3 dilution), and stained with Giemsa. In radioautograph smears of unfractionated BM and PB, as well as in radioautographs of E and EAC rosettes from fractionated BM and PB, 500 cells were counted and cells containing 6–10 grains over their nuclei were considered weakly labeled, while cells containing greater than 10 nuclear grains were considered heavily labeled.

Lymphoid cells less than 8 μm in diameter were called small lymphocytes and those greater than 8 μm in diameter were called medium to large mononuclear cells.

Blastogenic response of BM and PB lymphocytes to mitogenic stimulation. BM and PB lymphocyte blastogenic responses to various mitogens were assayed separately but simultaneously for each subject. Sucrose gradient separated BM and Hypaque-Ficoll separated PB cells were suspended for culture in MEM-S supplemented with 0.02 M L-glutamine, penicillin 100 U/ml, streptomycin sulfate 100 μg/ml, and 15% homologous AB serum (Antibodies Inc., Washington, D. C.). Cultures were done in microtiter plates (Cooke Laboratory Products, Cooke Engineering Co., Alexandria, Va.), as previously described (31). Each well contained 0.2 ml of cells in a concentration of 0.5×10^6 lymphocytes per ml. Quadruplicate cultures were incubated at 37°C in 5% CO_2 in air at 100% humidity. 10 μl of mitogen at various concentrations were added to each well. The mitogens used were: phytohemagglutinin (PHA) MR 68, lot K 4402 (Wellcome Reagents, Ltd., Beckenham, England), dose response curve from 0.5 to 10 μg/ml of culture; concanavalin A (Con A), lot 5,299 (Nutritional Biochemicals Corp., Cleveland, Ohio), dose response curve from 10 to 250 μg/ml of culture; and pokeweed mitogen (PWM), lot 180,690 (Grand Island Biological Co., Grand Island, N. Y.), 20 μl of a 1:100, 1:10, 1:5, or 1:2 dilution in distilled water per 0.2 ml of culture. Optimal stimulation for both BM and PB lymphocytes was found at 3 days of culture for PHA and Con A and at 5 days for PWM. 4 h before harvesting, 0.4 μCi of ^{3}HTdR (6.7 Ci/mM, New England Nuclear) was added to each well. The cells were collected from the wells onto fiberglass filters using a semi-automated microharvesting device. The filters were washed with 10% trichloracetic acid (TCA) and 95% ethanol and placed in 10 ml of Aquasol® (New England Nuclear). The TCA-precipitable radioactivity was counted in a liquid scintillation counter (Model LS-350, Beckman Instruments, Inc., Fullerton, Calif.). The arithmetic mean of the cpm of quadruplicate cultures was determined and the degree of stimulation was expressed where indicated either as the difference in cpm per 10^6 lymphocytes between stimulated and unstimulated (control) cultures (Δcpm), or as the stimulation index which is the ratio of cpm of stimulated or experimental cultures to control cultures (E/C).

Mixing of autologous PB with irradiated BM. To determine the maximal potential contribution of contaminating PB to the mitogen induced blastogenesis of BM lymphocytes, the following was done. In four subjects BM aspirates were performed. A volume of PB equal to the volume of BM aspirate was simultaneously drawn. The BM specimen was then irradiated with 10,000 rads from a ^{60}cobalt source. The nonirradiated PB was added to the irradiated BM cells, and the mixture was fractionated over a sucrose gradient, as described above, and then cultured in the presence of various doses of PHA. Hence, any blastogenesis noted would result from the small number of nonirradiated

PB lymphocytes in the irradiated BM specimen. Irradiated BM alone was run as a control.

Statistics. Statistical analysis of data was performed using Student's *t* test (32).

RESULTS

Mononuclear cell-enriched BM suspensions prepared from sucrose gradients. Sucrose gradient centrifugation resulted in a relatively pure mononuclear cell suspension (Fig. 1). Morphologic classification of BM mononuclear cells is quite difficult, particularly in microscopy involving rosettes and/or radioautography. Therefore, mature small, medium, and large lymphocytes, monocytes, immature lymphocytes and monocytes, and mononuclear stem cells were grouped for convenience into the category of mononuclear cells to distinguish them from the granulocyte and erythrocyte series in various stages of maturation within the BM. Unfractionated BM contained a mean (±SEM) of 14.2-(±1.6)% mononuclear cells. After sucrose gradient purification, cell suspensions from the upper layer of the gradient contained 69.8(±2.2)% mononuclear cells with a yield of 79.5(±5.8)% of the total mononuclear cells originally put on the gradient. The viability of cells after separation on sucrose gradients was always greater than 90%. Compared to this sucrose gradient technique, the Hypaque-Ficoll method is relatively inadequate in obtaining purified mononuclear cell suspensions from BM aspirates. Such preparations consistently contain up to 75% contamination with nonmononuclear cells, particularly those of the early granulocytic series such as myelocytes and metamyelocytes.

Contamination of BM aspirates with PB. The maximal percent contamination by volume of BM aspirates with PB in six subjects is shown in Table I. Despite meticulous care in drawing no more than 0.5–1.0 ml of BM aspirate, as well as demonstrating macroscopically visible bone spicules in each of the six aspirates, the samples were still contaminated with 67.8(±9.5)% by volume of PB. Because the cell density of the BM samples is so much greater than that of PB, the actual absolute number of mononuclear cells in the contaminating PB was only $1.1(\pm0.2)\times 10^6$, whereas the absolute number of mononuclear cells in the BM sample was $9.7(\pm1.5)\times 10^6$. Hence, when the number of mononuclear cells in the BM sample which were contributed by contaminating PB was divided by the total number of mononuclear cells in the BM sample for each specimen, the actual contamination of BM mononuclear cells with PB mononuclear cells was 13.7(±3.8)%.

TABLE I

*Maximal Contamination of BM Aspirate with PB**

Subject	cpm‡ per ml BM	cpm per ml blood	% volume contamination of BM aspirate with PB§
G. O.	5	18	28
B. W.	11	15	73
M. N.	18	30	60
S. N.	26	39	67
D. H.	20	24	83
A. P.	23	24	96
Mean	17.2	25.0	67.8
±SEM	±3.2	±3.5	±9.5

* BM aspirate performed simultaneously with drawing of PB in six subjects who had received ^{51}Cr-labeled autologous RBC.
‡ cpm of samples minus background.
§ cpm per ml BM/CPM per ml blood × 100 = % volume contamination.

TABLE II

Relative Proportions of Ig-Bearing Lymphocytes in BM and PB

	Percent lymphocytes bearing surface Ig		
	IgG	IgM	IgA
PB, *n* = 10*			
Mean	14.6	7.4	4.8
Range	6–21	4–13	0–9
±SEM	±1.5	±0.9	±0.9
BM, *n* = 10			
Mean	5.7	15.4	2.3
Range	2–10	7–24	0–7
±SEM	±0.8	±1.9	±0.7

* *n* is number of subjects studied.

Proportions of Ig-bearing lymphocytes in BM and PB. The relative proportions of lymphocytes bearing surface IgG, IgM, and IgA markers in the BM and PB of 10 subjects are shown in Table II. Lymphocytes bearing surface IgG predominate in the PB, while those bearing surface IgM predominate in the BM. There is only a small percentage of IgA bearing lymphocytes in the BM (2.3[±0.7]%).

Relative proportions of T and B lymphocytes as well as mononuclear cells without detectable surface markers in the BM. In attempting to determine as accurately as possible the proportions of BM lymphocyte populations, it was considered crucial that strict attention be paid to the contamination of the BM aspirate with PB lymphocytes since it was known from labeling studies (Table I) that an apparently adequate BM aspirate can be contaminated with up to 96% by volume of PB. Hence, maximal (100%) contamination by volume of PB was presumed in each of the BM aspirates. Simultaneous E and EAC rosette determinations were performed on PB and BM samples from 22 subjects. The mean (±SEM)% E and EAC rosette-forming lympho-

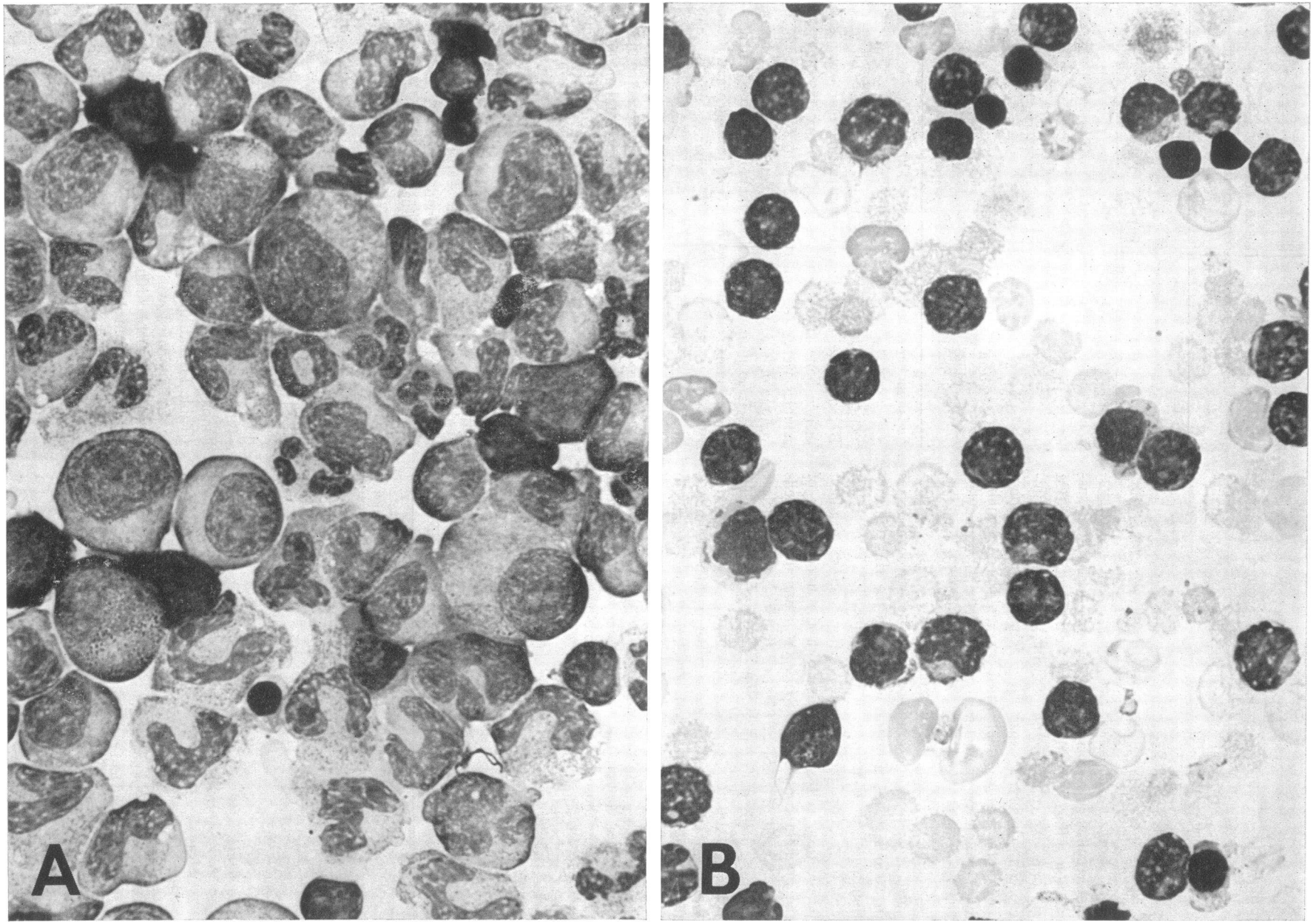

FIGURE 1 Fractionation of BM on sucrose gradients. A. Cytocentrifuge smear of unfractionated bone marrow. B. Cytocentrifuge smear of upper portion of gradient showing relatively pure mononuclear cell suspension (Magnification × 880).

cytes in the PB were 69.0(±2.3)% and 19.3(±1.0)%, respectively. The total number of PB E and EAC rosettes that could be contained in the volume of the BM aspirate in each individual was determined presuming maximal contamination by PB. This number was subtracted from the total number of E and EAC rosettes in the BM sample, and then the percentage of E and EAC rosettes in the BM after "correction" for PB contamination was calculated. These relative proportions in the BM of E and EAC rosette-forming cells, as well as lymphoid cells without detectable surface markers, are shown in Table III.

TABLE III

*Percentage of T and B Lymphocytes and Mononuclear Cells without Detectable Surface Markers in Normal Human BM**

Subject	T cells‡	B cells§	Cells without surface markers‖
	%	%	%
1	19	16	65
2	9	8	83
3	0	9	91
4	16	10	74
5	4	14	72
6	2	25	73
7	21	10	69
8	10	8	82
9	14	13	73
10	3	11	86
11	6	30	64
12	10	21	69
13	22	8	70
14	3	ND¶	—
15	6	30	64
16	23	23	54
17	0	ND	—
18	0	26	74
19	16	ND	—
20	0	ND	—
21	5	9	86
22	0	7	93
Mean	8.6	15.4	74.6
±SEM	±1.7	±1.9	±2.4

* Simultaneous determinations of total numbers of T and B lymphocytes in the PB and BM of each subject were performed. The maximal number of PB T and B lymphocytes which could possibly contaminate the volume of BM aspirate was determined and this amount was subtracted from the total number of T and B lymphocytes found in the BM aspirate. The percentages of BM T and B lymphocytes were then determined. Hence, the percentages shown are the minimal values based on correction for maximal contamination of BM aspirate by PB.

‡ SRBC rosettes.

§ EAC rosettes.

‖ Mononuclear cells lacking easily detectable T- or B-cell surface markers.

¶ Not done.

TABLE IV

Percentage of Labeled E and EAC Rosettes in BM and PB

	% of E rosettes labeled	% of EAC rosettes labeled
BM		
Subject 1	1.3	0.8
2	0.3	1.0
3	0.3	1.5
4	0.8	1.5
5	0.0	0.3
6	0.5	1.7
7	0.8	1.3
Mean	0.57	1.16
±SEM	±0.16	±0.19
PB		
Subject 1	0.5	0.8
2	0.5	0.3
3	0.0	0.0
4	0.3	0.0
5	0.3	0.3
6	ND*	ND
7	ND	ND
Mean	0.32‡	0.28§
±SEM	±0.09	±0.15

* Not done.

‡ No significant difference between BM and PB ($P > 0.2$, Student's t test).

§ Significant difference between BM and PB ($P < 0.01$).

Radioautographic studies in BM and PB. In vitro incubation of BM aspirates from 12 subjects for 1 h with ^{3}HTdR resulted in labeling of all cell types. After incubation of unfractionated BM with ^{3}HTdR, mononuclear cells were separated on sucrose gradients and it was found that 14.9(±2.3)% of the mononuclear or lymphoid cells were heavily labeled. Almost all of these were medium to large sized cells.

After incubation of PB with ^{3}HTdR in seven subjects, no labeling of the granulocytic series was seen. Only 0.57(±0.30)% of the mononuclear or lymphoid cells in the PB were labeled. Of these, labeled small lymphocytes were not found, and only medium or large mononuclear cells were labeled. Most of these were heavily labeled.

To compare the potential for spontaneous proliferation of T and B lymphocytes in the BM and PB, E and EAC rosette determinations were performed following incubation of BM or PB lymphoid cells with ^{3}HTdR and radioautographs were made (Fig. 2). Table IV lists

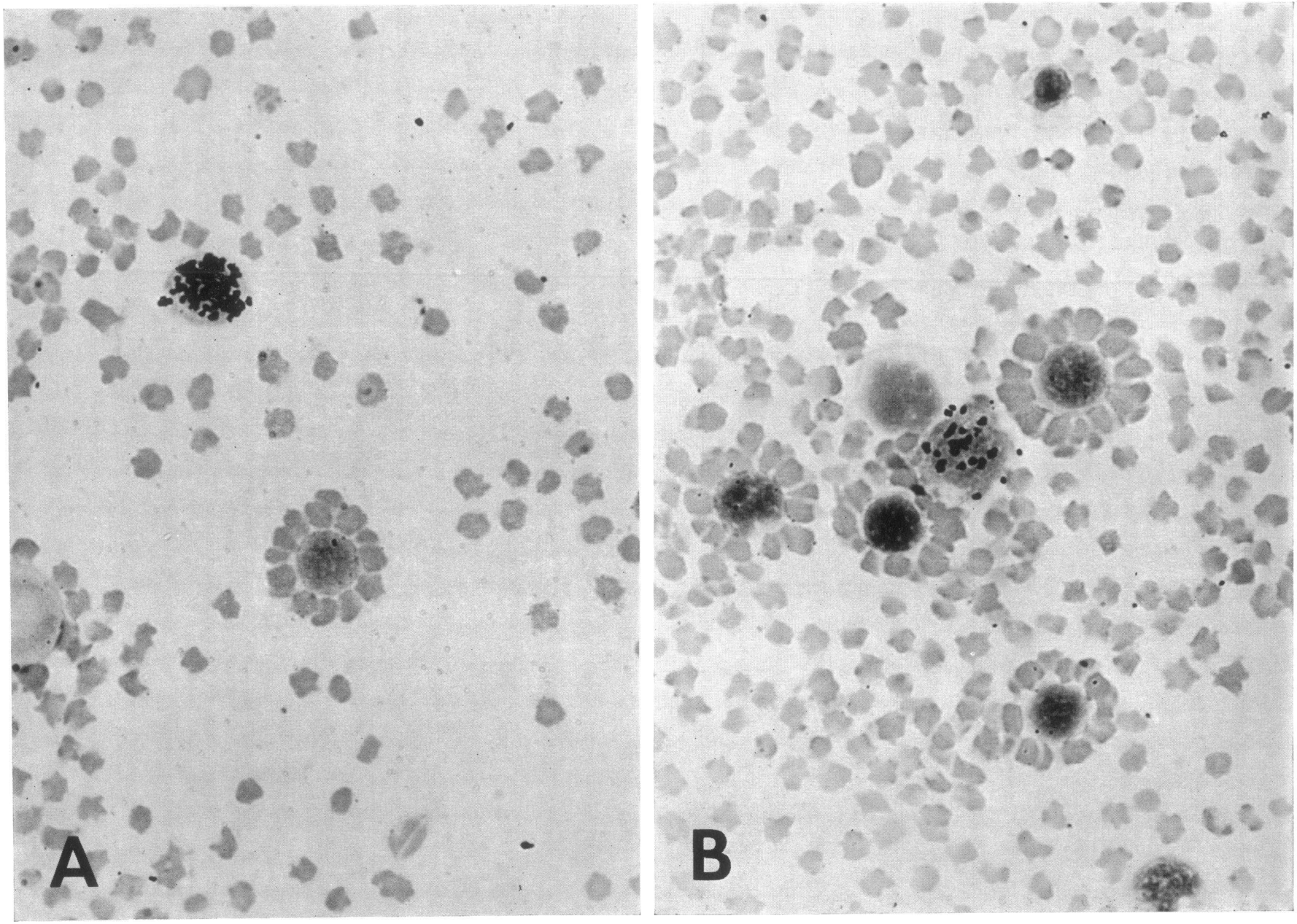

FIGURE 2 Combined lymphocyte rosette radioautographs done on lymphocyte-rich fraction of BM. A. Unlabeled E rosettes with heavily labeled mononuclear cell. B. Several unlabeled E rosettes with labeled and unlabeled mononuclear cells.

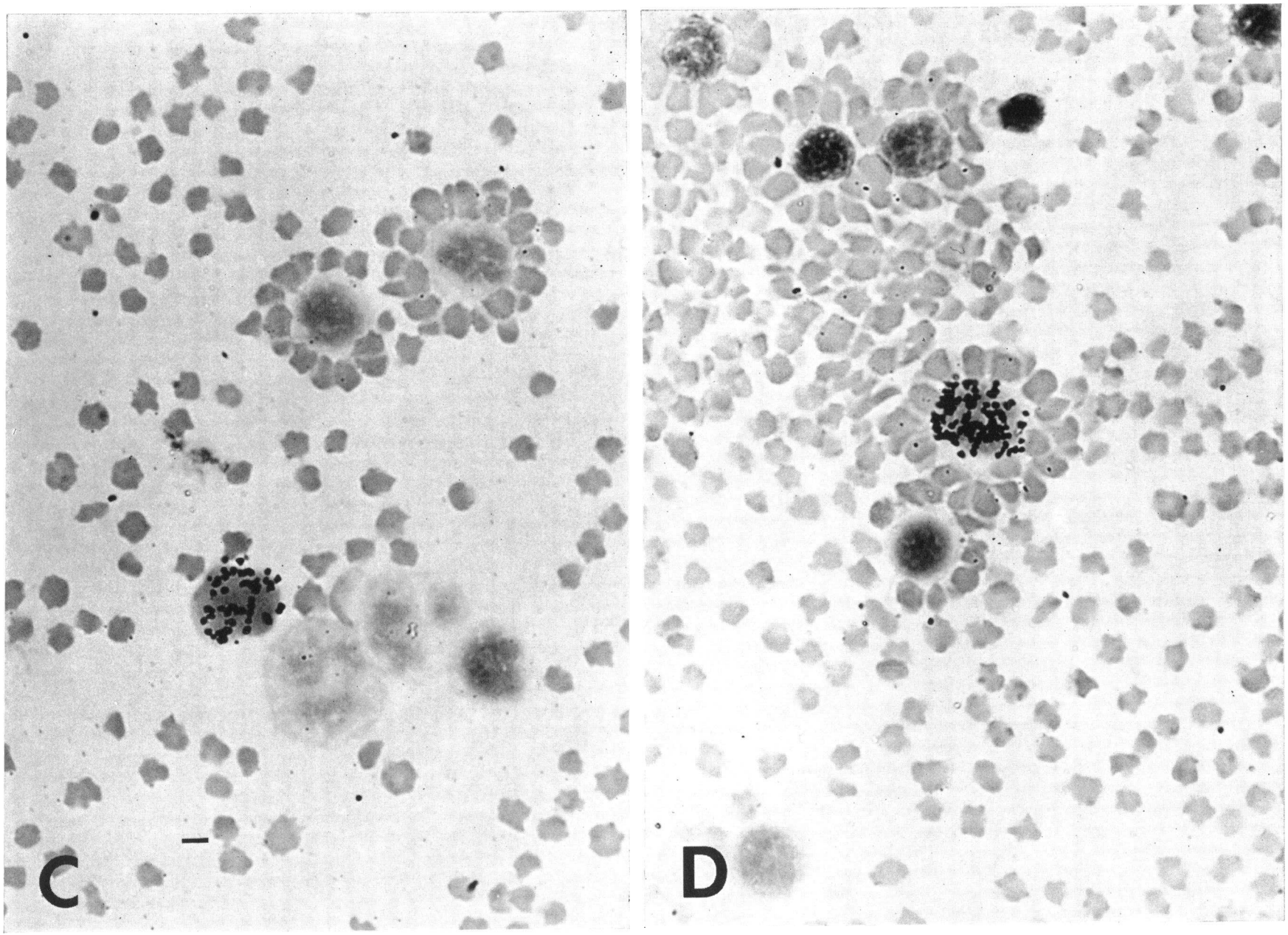

FIGURE 2 C. Unlabeled EAC rosettes with labeled and unlabeled mononuclear cells. D. Heavily labeled EAC rosette with several unlabeled EAC rosettes.

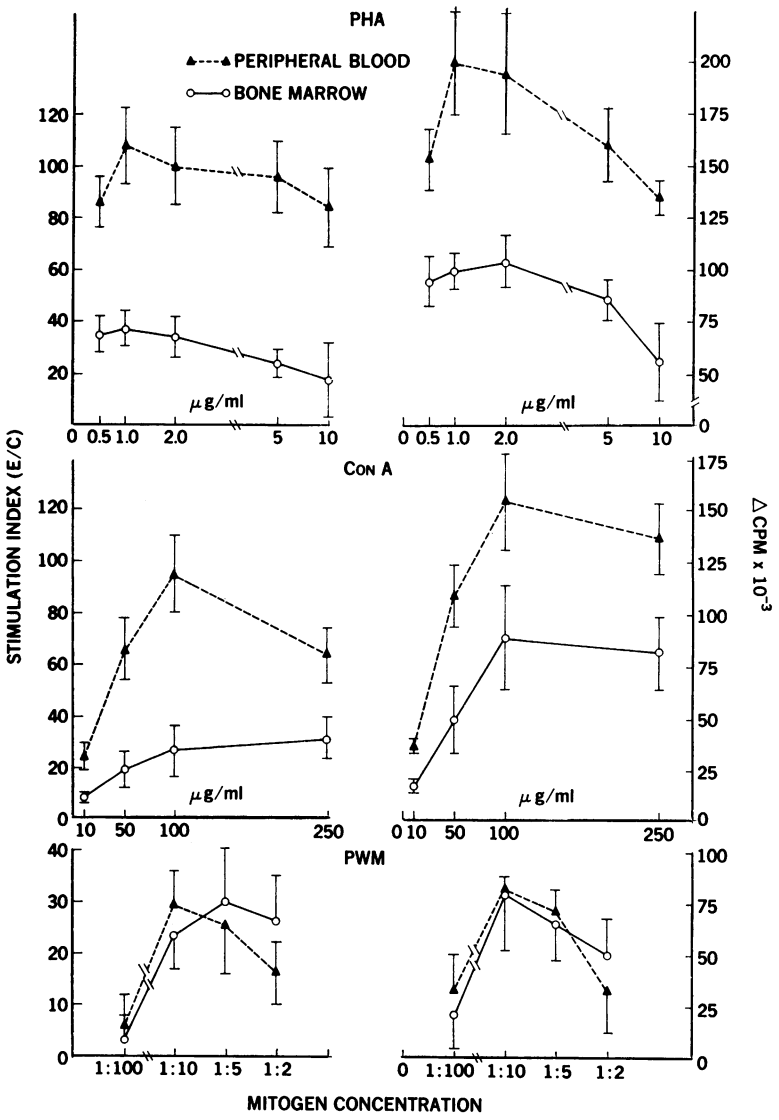

FIGURE 3 Relative blastogenic responses of peripheral blood and BM lymphocytes to PHA, Con A, and PWM. Points shown are the mean (±SEM) responses in 15 subjects. Responses are expressed both as the stimulation index (E/C) and the Δcpm.

the relative percentages of E and EAC rosettes in the BM and PB which were labeled. There is little difference in the percentage of labeled E rosettes in the BM and PB ($P > 0.2$, Student's t test) indicating that the proliferative capacity of these populations of lymphocytes whether in the BM or circulating in the PB is the same. On the other hand, EAC rosette-forming lymphocytes in the BM have a greater potential for spontaneous proliferation than do their counterparts in the PB ($P < 0.01$). As mentioned above, no labeled small lymphocytes were seen, and so all labeled E and EAC rosettes in the BM and PB were medium or large lymphocytes.

Mitogen induced blastogenic responses of BM and PB lymphocytes. Fig. 3 illustrates the relative responses of BM and PB lymphocytes of 15 subjects to in vitro stimulation with the mitogens PHA, Con A, and PWM. Dose response curves demonstrate clear-cut responses of BM lymphocytes to all three mitogens whether the data are expressed as the stimulation index or Δcpm. Of note is the fact that fractionation of BM lymphoid cells on sucrose gradients was essential to obtain such responses. Culture of unfractionated or Hypaque-Ficoll separated BM aspirates resulted in very poor responses to mitogens with stimulation indices of only 1–3 in most cases. In separate preliminary experi-

ments, separation of PB lymphocytes by the sucrose gradient technique did not noticeably alter the responsiveness to mitogens as compared to Hypaque-Ficoll separated PB lymphocytes. Of interest is the fact that PB lymphocyte responses to PHA and Con A were noticeably much greater than BM lymphocyte responses, while BM lymphocyte responses to PWM were as great as those of PB lymphocytes.

To rule out the possibility that lymphocytes from the small amount of PB contaminating the BM aspirates were responsible for the mitogen responses seen with the BM aspirates, irradiated BM aspirates were mixed with equal volumes of nonirradiated PB in four subjects (cf. Methods), and PHA responses were determined. Fig. 4 illustrates that PB lymphocytes contaminating the BM aspirate had a small but definite response to PHA when diluted in the BM lymphocytes. However, this response is far less than that seen with nonirradiated BM. Irradiated BM alone gave no response to PHA stimulation. Hence, the mitogen responses of BM aspirates seen in Fig. 3 may in part be explained by contaminating PB. However, this cannot nearly explain the entire mitogen response of the BM sample.

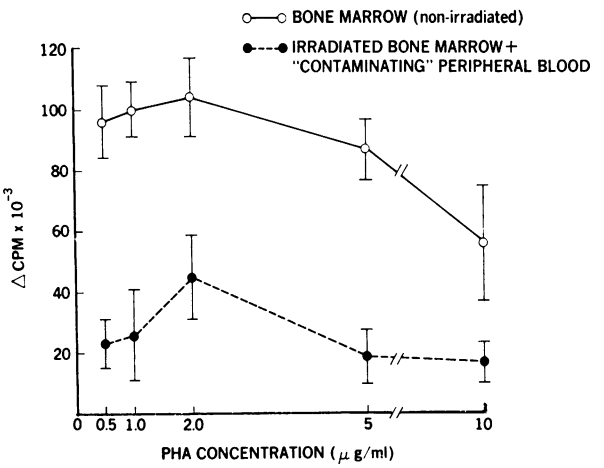

FIGURE 4 Effect of contaminating peripheral blood on PHA response of BM. Irradiated BM was mixed with an equal volume of nonirradiated peripheral blood. Data compare PHA response of normal BM with PHA response contributed by "maximal contamination" of aspirate by peripheral blood.

DISCUSSION

It has been firmly established that there exists in the lymphocyte pool within the BM of normal animals a small fraction (10% or less) of long-lived, recirculating lymphocytes which presumably are mature T lymphocytes (3, 4, 33). Their presence cannot be explained by blood within vascular channels in the BM, and they are contained within the BM parenchyma itself (3). These cells belong to a vastly larger total body recirculating lymphocyte pool which can migrate freely into and out of lymphoid compartments. A small fraction of these cells gain entrance to and take up normal residence in the BM (3, 4).

The concept of the accessibility of the BM parenchyma to the traffic of recirculating mature T lymphocytes is strengthened by the fact that manipulations such as corticosteroid administration which can alter the distribution of recirculating T lymphocytes results in an accumulation of these cells within the BM parenchyma (34–36). The demonstration of T cell function in BM cell suspensions is in keeping with the presence of a fraction of mature T lymphocytes (7–13). The use of linear sucrose density gradients to obtain highly purified BM lymphoid cell suspensions (22) in animal studies has allowed for greatly improved investigations of BM lymphocyte populations. This technique has recently been applied to the study of human BM in certain immunodeficiency diseases (15, 37) and was used in the present studies to obtain purified mononuclear cell suspensions from BM with relative high cell yields.

Because of the conflicting data with regard to the presence of mature T cells within human BM (14–20), it was considered essential that elements of artifact be controlled as much as possible in the present investigation. For this reason, BM aspirates were performed on a relatively large number of normal young adult volunteers. In addition, in the characterization of the lymphocyte populations of human BM, it is of utmost importance that the role of PB contaminating the BM aspirate be taken into account. The ^{51}Cr-labeled RBC studies demonstrated that despite meticulous care, considerable contamination by volume of PB occurs with every aspirate. Although the absolute number of lymphoid cells from the small volume of contaminating PB is small when compared to the absolute number of lymphoid cells in the BM aspirate, this nevertheless may significantly alter the proportion of a lymphocyte population which is considered to be within the BM when the true proportion is very small to begin with. The simultaneous determination of surface markers of PB and BM lymphocytes in the present study has allowed us to determine and correct for the precise amount of maximal possible contamination of BM by PB lymphocytes. Hence, the corrected proportion of 8.6(±1.7)% for E rosettes and 15.4(±1.9)% for EAC rosettes in the BM of these particular subjects cannot be attributed to PB contamination. The percentage of T lymphocytes in the BM in this study is remarkably close to the figure of 8(±5)% of BM lymphocytes bearing surface T-cell markers in mouse studies in which BM cell suspensions were also obtained by sucrose gradient separations (38).

In addition, the proportions of T and B lymphocytes, as well as lymphoid cells without detectable surface markers found in the BM of normal individuals (Table III), are quite similar to the data reported in BM studies of a group of children in long-term (3–8 yr) continuous remission from acute lymphoblastic leukemia (20). In contrast, our findings of 8.6% E rosettes in BM is higher than the 0–2% reported in human studies in which bovine serum albumin gradient separations were used (16, 17).

Previous studies have shown that contrary to the PB in which there is a greater proportion of IgG-bearing lymphocytes than IgM-bearing lymphocytes, the reverse is true in BM, i.e., there is a significantly greater proportion of IgM-bearing lymphocytes (20, 37). This same pattern was noted in the present studies. The slight discrepancy between the proportion of Ig-bearing and EAC-rosetting lymphocytes in the BM may be explained by one or more of several factors including a lack of comparable sensitivity of the techniques used, the recognized difficulty in distinguishing lymphocyte from nonlymphocyte mononuclear cells in the BM, and the fact that lymphocytes in various stages of development may preferentially express or fail to express one or more of their potential markers (39).

In addition to a fraction of lymphocytes bearing surface T-cell markers, a definite response to mitogenic stimulation was present in the BM lymphocyte fraction (Fig. 3). Of interest is the fact that there was a markedly greater responsiveness of PB lymphocytes to PHA and Con A than BM lymphocytes. Yet, the response of BM lymphocytes to PWM was proportionately greater than to PHA and Con A when compared with PB lymphocytes. In fact, the response of BM lymphocytes to PWM was equal to that of PB lymphocytes. These findings demonstrate: (*a*) that there is a definite fraction of PHA and Con A responsive lymphocytes in the BM of normal individuals; and (*b*) the population of BM lymphocytes responsive to PWM is proportionately greater than the population responsive to PHA and Con A when compared to PB. Hence, functionally distinct subpopulations of lymphocytes as measured by differential response to mitogenic stimulation are present both in the PB and BM of normal individuals, but in measurably different proportions in the two compartments.

The problem of contaminating PB lymphocytes must always be considered in the evaluation of the mitogen responsiveness of BM lymphocytes. As shown, there is a small but definite fraction of BM lymphocytes bearing surface T cell markers over and above those attributable to contaminating PB. It is difficult to determine the relative contributions of BM lymphocytes and contaminating PB lymphocytes to the mitogen responsiveness of the fractionated BM aspirates shown in Fig. 3. For this reason experiments were performed mixing nonirradiated PB with irradiated BM (Fig. 4). These studies clearly showed that although contaminating PB lymphocytes give a detectable PHA response, they are not sufficient to account for the PHA responsiveness of the BM lymphoid cells. One can reasonably assume that the PHA responsiveness of BM lymphocytes within the BM parenchyma is due to the small fraction of these cells bearing the surface T-lymphocyte marker. However, it cannot be ruled out by the present techniques that other lymphocyte subpopulations which do not form E rosettes are also contributing to the PHA responsiveness of the BM lymphoid cells.

Inasmuch as animal studies (3, 4, 33) have shown that the T lymphocytes found in BM are long-lived recirculating lymphocytes which have entered the BM parenchyma from the recirculating lymphocyte pool, one would expect the maturity and proliferative capacities of T lymphocytes in the BM and PB to be similar. Although it is extremely difficult by presently available techniques to accurately determine the relative states of functional maturation of lymphocyte subpopulations, an attempt was made to address the question of relative proliferative capacities by comparing the spontaneous DNA synthesis (28–30) of T and B lymphocytes in the PB and BM. Thus, radioautographs after in vitro incubation with ^{3}HTdR were done on unfractionated PB and BM, as well as simultaneous rosette radioautographs on fractionated PB and BM lymphocytes. In unfractionated PB, no labeled cells of the granulocytic series were seen nor were labeled small lymphocytes seen. Labeling was limited to a small fraction of medium to large sized mononuclear or lymphoid cells. This is in close agreement with other labeling studies in human PB (29, 30). In unfractionated BM, it was noted in the present experiments that after 1 h of in vitro incubation with ^{3}HTdR, cells of all series were labeled. After fractionation of labeled BM lymphoid cells, 14.9(±2.3)% of the mononuclear cells were labeled. This figure is in close agreement with the proportion of guinea pig BM lymphoid cells labeled 1 h after in vivo administration of ^{3}HTdR (40).

The similarity in proliferative capacities between T lymphocytes in the PB and BM (Table IV) is compatible with, but in no way proves that, the T lymphocytes in these compartments are from the same population. If, however, they are the same population of cells, this would be consistent with animal data which have shown that the T lymphocytes found in the BM parenchyma are part of the same recirculating pool of T lymphocytes found in the PB (3, 4, 33). In addition, the relatively greater proportion of EAC-rosetting B lymphocytes with proliferative capacity in the BM as compared to PB is compatible with the BM origin of

these cells (1), as well as with the loss of a certain degree of proliferative potential as they leave the BM and enter the PB. However, these data do not rule out the possibility that EAC-rosetting lymphocytes in the PB may be a distinct population of cells from the EAC-rosetting lymphocytes in the BM and may not result from a further maturation of these cells originally present in the BM.

Hence, these studies have shown that in addition to a B cell compartment, the normal human BM contains a small but definite fraction of T lymphocytes. These cells are probably part of the larger recirculating pool of mature T lymphocytes which have gained entrance to and reside normally in the BM. More precise characterization of lymphocyte subpopulations in the human BM will be essential to further understanding of various immunodeficiency diseases, such phenomena as the graft-versus-host reaction associated with BM transplantation, as well as compartmentalization of lymphocytes in certain disease states and during various chemotherapeutic regimens.

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