PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 174, 153–159 (1983)

Studies of BFU-E in Flask Cultures of Human Peripheral and Cord Blood Cells[1] (41718)

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Abstract. Early erythroid progenitors (the burst-forming units-erythroid or BFU-E) from human peripheral and cord blood mononuclear cells were maintained in flask culture for 2 weeks without added erythropoietin (Epo) or erythroid potentiating activity (EPA). These cultures did not develop adherent cell layers and did not support the more mature erythroid colony-forming unit (CFU-E). Samples removed at intervals from these flask cultures were assayed for BFU-E recovery in a plasma clot system in response to a range of Epo doses and to added EPA with time in flask culture. The BFU-E recovered showed increased proliferative capacity and decreased responsiveness to Epo and EPA. These results indicate selection of more primitive erythroid progenitor cells under the conditions described. Peripheral and cord blood mononuclear cell cultures provide a flexible and accessible approach to *in vitro* studies of human erythropoiesis. Comparative studies with long-term marrow cultures should help to elucidate the role of adherent cells and humoral factors in erythroid differentiation.

Continuous *in vitro* culture systems for human erythroid progenitor cells have been difficult to establish. A method originally described by Dexter and co-workers (1) in which murine hematopoietic precursors are maintained in suspension over an adherent feeder layer has been adapted for human marrow cultures (2–4). However, species-specific differences in the adherent feeder layer alter the patterns of progenitor cell growth and maintenance. Human marrow cultures produce high levels of colony stimulating factor (CSF) compared to similar cultures of mouse or *Tupaia* (the tree shrew) (5). This CSF produced *in vitro* may be responsible in long-term human bone marrow cultures for the eventual domination of the adherent layer by macrophages and for a corresponding decrease in the numbers of hematopoietic precursors which can be recovered from the cell suspension. Immature erythroid progenitors (the burst forming unit-erythroid or BFU-E) have been especially difficult to recover from such cultures. BFU-E were initially observed for about 2 weeks in human cultures containing an adherent layer compared to BFU-E from mouse bone marrow which have been maintained continuously for more than 2 months in flask culture.

Recent reports indicate that layering a second autologous bone marrow sample over a preestablished adherent layer makes it possible to maintain human marrow BFU-E for up to 5 weeks in culture (4, 6). However, these cultures were even more successful for the maintenance of the more differentiated erythroid progenitor (the colony-forming unit-erythroid or CFU-E) and for the granulocyte–macrophage precursor (or CFU-C), each of which could be observed for longer than 2 months. Eaves and colleagues (3) obtained preferential recovery of primitive erythroid precursors by removing the adherent cells from human marrow cultures. They also found that an irradiated feeder layer would support both CFU-E and two subclasses of BFU-E for 2 to 3 weeks in suspension. These results suggest that primitive human erythroid progenitor cells do not require an adherent layer for maintenance in suspension culture and that the most primitive erythroid procursors in bone marrow may be more successfully cultured without the presence of adherent cells. We have taken these studies one step further by initiating flask cultures from peripheral blood and cord blood cell preparations. We have found that suspension cultures of human BFU-E can be established from stem cells circulating in the peripheral blood of normal adults and in cord blood of infants.

[1] Supported in part by grants from the National Science Foundation and the National Institutes of Health.

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0037-9727/83 $1.50

These human erythroid progenitors can be maintained in suspension culture for at least 2 weeks without the addition of exogenous erythropoietin and without the presence of an adherent feeder layer. We have observed as well a preferential recovery of increasingly primitive erythroid progenitors with time in culture. Addition of erythroid potentiating activity (EPA) to the BFU-E assay mixture permitted further characterization of the erythroid precursor population in peripheral blood suspension cultures.

Information on the recovery of erythroid progenitors from cord blood or peripheral blood mononuclear cell (PBMC) suspension cultures complements that obtained from analyses of long-term bone marrow cultures. Differences exist in the extent to which these cultures will support the maturation of erythroid precursors. Comparative studies using both peripheral blood and bone marrow flask cultures may make it possible to identify the cell types and cell-to-cell interactions responsible for these differences in *in vitro* erythropoiesis.

Materials and Methods. *Establishing cultures.* Peripheral blood samples were obtained from normal, healthy volunteers after appropriate informed consent. Normal cord blood samples were obtained from the Yale–New Haven Hospital newborn nursery. Both types of blood samples were collected sterilely and were immediately added to minimum essential medium (MEM) containing 2% FCS and preservative-free heparin. Mononuclear cell fractions were obtained by Ficoll–Hypaque density centrifugation of these prepared samples. On "Day 0" of our experiments, mononuclear layer cells were either plated directly into plasma clot cultures or prepared for suspension culture. For growth in tissue culture flasks, mononuclear cells were rinsed two to three times in MEM with 2% FCS before being suspended at a density of $1–2 \times 10^7$ cells/ml in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 units of penicillin, 100 μg/ml of streptomycin, 2 m*M* L-glutamine, 0.1 m*M* nonessential amino acids, and 1 m*M* sodium pyruvate. These suspension cultures were maintained in plastic T-25 flasks (Corning) at 37°C in a humidified atmosphere of 5% CO_2 in air. Feeding was done at each time point by removing 1 to 2 ml of the medium containing the suspended cells and replacing it with an equal volume of fresh medium. Cells were tested for viability by trypan blue exclusion counts at each time point.

BFU-E assay. The presence of erythroid progenitor cells was assayed at five time points over a 2-week period. Aliquots of medium containing suspension cells were removed from the flasks as described above and the mononuclear cell fraction isolated again by Ficoll–Hypaque density centrifugation. Assays were performed in plasma clot culture using Tepperman's method (1). The standard medium employed for the plasma clots was α-MEM containing beef embryo extract, bovine serum albumin, α-thioglycerol, L-asparagine with calcium chloride (each supplement at a concentration of 10%), 30% fetal calf serum, and a range of erythropoietin (Epo) concentrations from 10–40 μl/ml (1 to 4 IU/ml). A control without erythropoietin was routinely used. One-tenth milliliter of this media, containing 10^6 cells/ml, was placed into a series of sterile plastic microwells. The cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. The human urinary erythropoietin used was collected and concentrated by the Department of Physiology, University of Northeast Argentina and further processed to minimize endotoxin activity by the Hematology Research Laboratory, Children's Hospital of Los Angeles.

After 14 days the plasma clots were removed from incubation, fixed with glutaraldehyde, and stained with dimethoxybenzidine and hematoxylin. The number of erythropoietic bursts was determined under 100× magnification. Only clusters of three or more aggregates of 8 to 49 benzidine-positive cells or single clusters of 50 or more benzidine-positive cells were scored as BFU-E-derived colonies.

In the final set of BFU-E assays, erythroid burst enhancing activity was added to one-half of the plasma clots which again were supplemented with a range of erythropoietin concentrations from 1 to 4 IU/ml. Erythroid potentiating activity was provided to the assay mixture in the form of conditioned medium obtained from a human T-cell culture (the Mo cell line). Golde *et al.* have reported the enhancement of proliferation of human erythroid progenitor cells *in vitro* by this T-cell-conditioned medium (8). The Mo cell-conditioned medium (a gift of Dr. David W. Golde) was added to the assay mixture at a

concentration of 50 μl/ml as recommended for maximal erythroid burst-promoting activity.

Conditioned media effects on suspension culture. The effect of this T-cell-conditioned media on the growth of the total mononuclear cell population in suspension was also tested. A peripheral blood cell preparation prepared for suspension culture was divided in half and maintained in two flasks, one with and one without a 10% supplement of T-cell-conditioned media. Total nucleated cell counts were determined at five time points over a period of 3 weeks.

Morphologic studies. Each flask culture was examined daily on an inverted microscope under 10 and 20× magnification for any observable changes in the cultures. Characteristics of the culture were noted with respect to populations of individual suspension cells, suspension cell aggregates, and adherent cells. Adherent cells were defined as cells which would not move freely when the flask was shaken gently and which showed a two-dimensional flat appearance in the plane of the flask surface.

Aliquots of 0.1–0.2 ml of media containing suspension cells were removed from the flask culture for cytocentrifuge preparations (Shandon Instruments). Slides were stained with benzidine/hematoxylin or with Wright's stain and were examined under 40 and 100× magnification to determine the type of cell present in the cultures.

Results. At Day 0 all of the cord blood and peripheral blood flask cultures contained a dense population of individual suspension cells. After 2 days in culture the mononuclear cell population consisted of many discrete rounded clusters which were not attached to the flask surface as well as a background of individual suspension cells. Although a few adherent cells were observed in each flask, no adherent layer formed in any of these mononuclear cell cultures. Even cultures grown for 6 weeks without being depopulated for BFU-E assays did not show signs of developing foci of adherent cells. In contrast, flask cultures of human bone marrow fed with the same media under the same culture conditions in the past have consistently developed adherent layers.

Morphologic composition of the flask cultures was determined by examining cytocentrifuge preparations on Days 7 and 14. On both days the suspension cultures were composed of 95% mononuclear cells. The remaining 5% of the cells were granulocytes. None of the nucleated cells were benzidine positive.

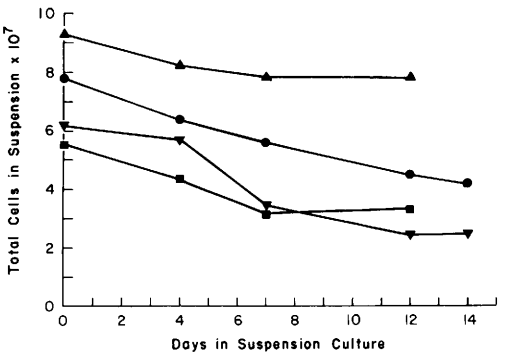

FIG. 1. Survival of peripheral blood (▲, ●, ■) and cord blood (▼) mononuclear cells in suspension cultures maintained without added erythropoietin or EPA. The raw data are plotted after correction for the removal of 10^7 cells for BFU-E assays at each time point.

Although the total nucleated cell count for the cord blood and peripheral blood flask cultures fell off dramatically over the culture period, 50 to 75% of the cell loss in suspension culture was due to removal of cells for plasma clot BFU-E assays. Cell counts corrected for depopulation (Fig. 1) indicate that the total cell population after 2 weeks in culture is approximately two-thirds the size of the initial population.

In three experiments with Day 0 (i.e., fresh) cord blood and five with Day 0 peripheral blood samples we recovered an average of 103 ± 2 BFU-E/10^6 mononuclear cells plated in the presence of 2 IU Epo/ml. This value is in the range of those reported in the literature for Day 0 preparations of both human peripheral blood mononuclear cells (9, 10) and human bone marrow buffy coat cells (3, 4, 6) assayed for BFU-E in the presence of 2 IU/ml of Epo.

In all of the peripheral blood and cord blood flask cultures erythropoietin burst-forming units could be assayed after 12 to 14 days of culture. The pattern of survival of BFU-E is shown for several different experiments in Table I. In the initial series of experiments we observed an increase in the total number of BFU-E after 4 days of suspension culture (Fig. 2). In several later experiments for which the serum used in flask media and plasma clot

TABLE I. BFU-E/FLASK IN THREE PERIPHERAL BLOOD EXPERIMENTS

	Experiment		
	1	2	3
Total cell input	6.2×10^7	9.3×10^7	7.9×10^7
BFU-E/flask[a]			
Day 0	5,500	13,000	5135
Day 4	10,800	20,200	2709
Day 7	3,000	12,300	1610
Day 12	1,200	7,400	321
Day 14	1,000	NA[b]	0

[a] Not corrected for depopulation effects.
[b] NA: not available.

cultures came from a different serum lot, we observed no increase in BFU-E number over a 14-day period in suspension culture (Fig. 3). No CFU-E-derived colonies were observed in any experiment.

Exogenous erythropoietin was not required in the flask cultures for the maintenance of BFU-E. However, erythropoietin was always required for the differentiation (in plasma clot culture) of these early erythropoietic precursors. The BFU-E precursors withdrawn from the flasks for plasma clot assays over a 2-week period required increasingly higher concentrations of erythropoietin for differentiation into erythroid colonies. In the experiments in which a proliferative burst of BFU-E was observed after 4 days in culture, the requirement for higher Epo doses to achieve maximal assay of BFU-E was observed after 1 week in culture. By Day 12 of flask culture, BFU-E from these flasks would not respond to 1 IU/ml Epo and responded only slightly to 2 and 4 IU/ml Epo. This change in the erythropoietin dose response was even more striking in the later experiments (see Fig. 3). The extent to which this effect could be altered by the addition of erythroid potentiating activity to the assay system was then tested.

Figures 3A and B demonstrate Day-14 BFU-E recovery in plasma clot cultures containing 0, 1, 2, or 4 units Epo/ml without and with the addition of erythroid burst-promoting activity. BFU-E precursors from Day-0 and Day-4 flask cultures differentiated in the presence of as little as 1 unit Epo/ml whether or not erythroid BPA was added. However, after 7 days in suspension culture the precursor cells required 2 units Epo/ml for differentiation without EPA and by Day 12 required 4 units Epo/ml for significant burst formation with or without EPA. Addition of erythroid burst-promoting activity to the plasma clot assay system caused a threefold increase in BFU-E colony formation at Day 0 and a twofold increase after 4 days of flask culture. However, as seen in Fig. 3B, EPA did not prevent the decrease in BFU-E responsiveness to erythropoietin. The population of primitive precursor cells maintained in suspension culture is significantly less responsive to EPA as well as to erythropoietin after 2 weeks in suspension culture.

In one experiment we examined whether the T-cell-conditioned medium, in addition to enhancing the cloning efficiency of erythroid BFU-E, might also cause a general stimulation of PBMC growth *in vitro*. As seen in Fig. 4, PBMC did not proliferate when T-cell-conditioned medium was added to the flask. Addition of this medium actually reduced the mononuclear cell counts by 15% of the control after one week and by 25% after 3 weeks of suspension culture in contrast to its positive effect on BFU-E proliferation in plasma clot cultures.

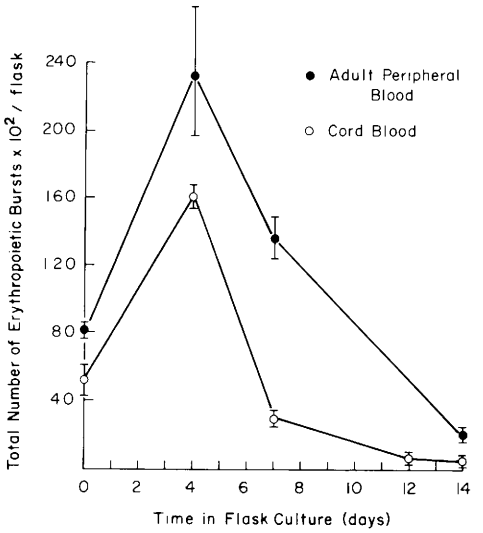

FIG. 2. Recovery of BFU-E from flask cultures in two experiments representative of the initial series in which an increase in the number of early erythroid progenitor cells was observed after 4 days of suspension culture. Each point represents the mean number of bursts counted in four identical plasma clot assays containing 2 units Epo/ml. Two additional cord blood and four peripheral blood studies showed a similar pattern of BFU-E recovery.

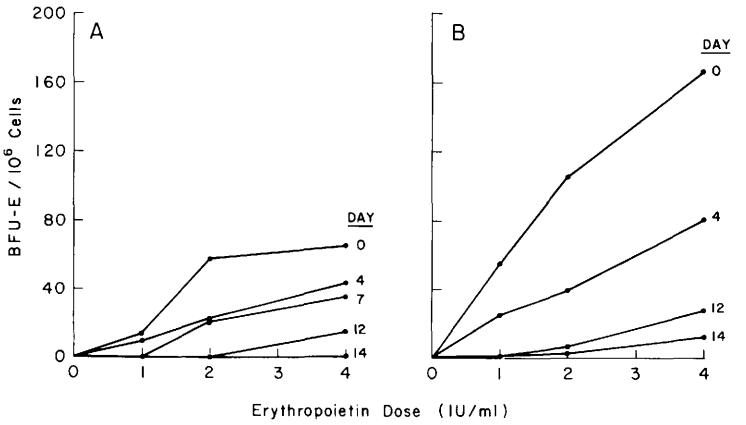

FIG. 3. Comparison of erythropoietin dose response for BFU-E with (B) and without (A) erythroid potentiating activity in the assay mixture. For this experiment peripheral blood mononuclear cells from a single individual were assayed for BFU-E recovery in the presence and absence of EPA. (A) demonstrates the pattern of BFU-E recovery when no burst of progenitor cell growth occurs early in flask culture.

Discussion. Long-term *in vitro* studies of erythroid differentiation have utilized cultures established almost exclusively from bone marrow preparations. Our experiments indicate that human peripheral blood and cord blood mononuclear cells can also be maintained in flask culture for the study of erythroid progenitor cells. Literature data together with our results show that the same range of BFU-E counts (80 to 500/10^6 cells plated) are recovered from "Day 0" preparations of cord blood, peripheral blood (9, 10), or bone marrow (3, 4, 6, 10) tissue. Cultures established from these different sources can thus be compared for characteristics in the *in vitro* development of the erythroid precursor population. Such studies may elucidate some of the site-specific mechanisms in the regulation of erythropoiesis.

An important similarity to long-term bone marrow cultures which we have noted in our characterization of peripheral and cord blood flasks cultures is the recovery of less differentiated erythroid precursors in the absence of erythropoietin and of an adherent feeder layer. Both mouse and human bone marrow studies have shown that the more differentiated erythroid progenitor, the CFU-E, require erythropoietin for survival in long-term culture and that CFU-E are more sensitive to erythropoietin than BFU-E (8, 10, 11).

Studies have also shown that BFU-E from marrow preparations and from Day 0 human peripheral blood preparations will respond to lower doses of erythropoietin in the presence of a factor found in serum or conditioned media (8, 10) which is referred to as burst promoting activity (11), erythroid enhancing activity (12), or erythroid potentiating activity (10). Our data confirm and extend these observations to peripheral blood and cord blood mononuclear cell preparations which have been maintained in flask culture.

Results from our experiments which tested the effects of erythroid potentiating activity on peripheral blood cells prior to long-term flask culture support the observation by Hamburger (10) that T-cell-conditioned medium

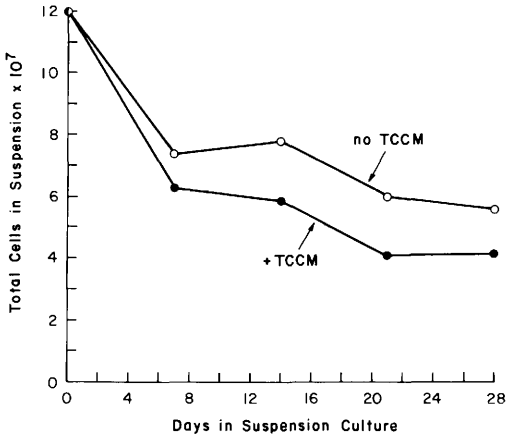

FIG. 4. Survival of the total mononuclear cell population in peripheral blood suspension culture with and without T-cell-conditioned media added to the flasks.

increases the number of BFU-E even when erythropoietin is not growth limiting. The equivalent points from our Fig. 3 would be the Day 0 points over a range of erythropoietin concentrations. Figure 3A indicates that the Epo is no longer growth limiting between 2 and 4 IU/ml for plasma clot cultures set up on Day 0 (i.e., without long-term flask culture). Yet, T-cell-conditioned medium in parallel colony assays causes a dramatic increase in BFU-E number even in the presence of 4 IU of Epo.

We have observed that Day-14 BFU-E but no CFU-E can be recovered from cultures maintained in flasks for 2 weeks without added erythropoietin or erythroid burst promoting activity. We have also observed that under these conditions the maintenance of mononuclear cells in suspension cultures selects for increasingly primitive erythroid progenitors. A consistent finding in these experiments was an increase in the erythropoietin required for maximal burst formation with increasing time in flask culture. After 2 weeks of suspension culture the BFU-E recovered were three- to fourfold less sensitive to erythropoietin than those assayed at Day 0.

Addition of an erythroid potentiating activity to the assay mixture caused a twofold increase in the recovery of BFU-E from flask cultures at Days 0 and 4. After 2 weeks of suspension culture, when no BFU-E growth could be observed in the presence of 4 IU/ml erythropoietin, the addition of erythroid potentiating activity to the assay mixture permitted recovery of early BFU-E from these flasks. As observed in earlier studies (10, 11), this erythroid enhancing activity lowered the threshold of erythropoietin required for burst formation. Thus erythroid potentiating activity in the assay mixture compensated partially for the decreased sensitivity to the erythropoietin observed in older suspension cultures. However, the combined effect of erythropoietin and erythroid potentiating activity in the assays ultimately accentuated the observed shift in the BFU-E population toward a more primitive erythroid cell.

We have shown that the simple addition of erythroid potentiating activity, in the form of T-cell-conditioned medium to a flask culture of peripheral blood mononuclear cells, does not cause a general stimulation of the total mononuclear cell population. This result underscores the positive selective effect of erythroid potentiating activity on BFU-E growth in plasma clot culture and also indicates that T-cell-conditioned medium does not cause nonspecific enhancement of cell division in culture. The observation that this burst-promoting factor causes a decrease in total mononuclear cell counts may reflect more rapid precursor maturation in the presence of erythroid potentiating activity to stages which, for survival, require pathway-specific factors (e.g., erythropoietin) not present in the flask medium. The presence of colony-stimulating factor in T-cell-conditioned medium (8) may also affect the maturation of nonerythroid cells in the flask cultures in a similar manner. This might then be reflected in a reduction in the numbers of total viable cells in culture over a period of 2 weeks.

Peripheral and cord blood suspension cultures without supplements of erythropoietin or erythroid potentiating activity may be comparable to cultures of fractionated nonadherent human bone marrow cells in which preferential recovery of the most primitive erythroid cells has been reported (3). Although our flask cultures contained an occasional adherent cell, none of the suspension cultures developed the adherent cell layer so typical of long-term bone marrow cultures. The use of fetal calf serum rather than horse serum in our flask culture medium may prevent some adherent cells from attaching to the flasks. However, we have observed that preparations of buffy coat cells from bone marrow will develop adherent layers containing fibroblast and endothelial-like cells at the same densities and under the culture conditions described for our peripheral and cord blood suspension cultures. This lack of development of any adherent layer in the peripheral blood mononuclear cell cultures should permit useful comparisons to long-term marrow cultures in defining the respective roles of adherent and nonadherent cells in erythroid differentiation.

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Received November 5, 1982. P.S.E.B.M. 1983, Vol. 174.