HUMAN ERYTHROID BURST-FORMING UNIT: T-CELL REQUIREMENT FOR PROLIFERATION IN VITRO*

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Recent studies of human erythropoiesis in vitro have provided evidence that the common hematopoietic stem cell (CFU-S)[1] differentiates to a primitive erythroid precursor with a high proliferative capacity. This precursor, known as the erythroid burst-forming unit (BFU-E), was described by Stephenson et al., Axelrad et al., and by Iscove et al. in murine marrow (1-4). The colony formed by a single BFU-E has a unique morphology characterized by multiple subcolonies, the number and size of which are functions of the erythropoietin concentration in the culture medium (5, 6).[2] BFU-E colonies with very low hemoglobin content in subcolonies begin to appear in plasma clot cultures of human marrow at about day 7 and reach full maturity of cell number and hemoglobinization by day 12-14 of incubation (6).[2] We assume, therefore, that during the first 7 days of culture, single BFU-E divide several times. The daughter cells form the nidus of each subcolony (7). Then each of the daughter cells becomes committed to further proliferation and hemoglobin synthesis, both of which occur over the next 7 days. After 7 days, the growth characteristics of the committed BFU-E subcolonies are very similar to the growth characteristics of the more familiar single erythroid colony-forming units observed in human marrow cultures. These colonies, known as CFU-E, grow to full maturity and hemoglobinization in 7 days. Their size and number are also proportional to the erythropoietin concentration in the culture medium (8). Both BFU-E and CFU-E are deficient in various clinical disorders of erythropoiesis including Diamond-Blackfan[2] (9, 10) and aplastic anemia (11). It appears likely, though complete proof is lacking, that BFU-E give rise to CFU-E

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[1] Abbreviations used in this paper: BFU-E, erythroid burst-forming unit; CFU-S, common hematopoietic stem cell; CSA, colony-stimulating activity; EAC, antibody-coated sheep erythrocyte. IU, international units; SCID, severe combined immune deficiency.

[2] D. G. Nathan, B. J. Clarke, D. G. Hillman, B. P. Alter, and D. E. Housman. Erythroid precursors in congenital hypoplastic Diamond-Blackfan anemia. J. Clin. Invest. In press.

which, in turn, are the precursors to the recognizable marrow erythroid cells.

Recent studies in our laboratories and elsewhere have shown that the BFU-E, though predominantly a marrow cell,[2] is not restricted to the marrow cell population. BFU-E can be detected in plasma clot cultures of mononuclear cells derived from peripheral blood (5, 12).[2] This fact provides an opportunity to define the surface characteristics of BFU-E and to evaluate possible interactions of various blood mononuclear cells in the development of the hemoglobinized colonies.

In this communication, we provide evidence that BFU-E reside in the null cell fraction of human peripheral blood mononuclear cells, but their proliferative capacity and erythroid differentiation in culture are dependent upon erythropoietin and a helper effect derived from the addition of T cells to the null cell population. Both isogeneic and allogeneic T cells can function in this helper role, and a soluble product of peripheral blood nucleated cells incubated in the presence of tetanus toxoid can substitute for intact T cells in this in vitro model of erythropoiesis.

Materials and Methods

Mononuclear cells were separated from fresh blood anticoagulated with preservative-free heparin at 10 U per ml. The separation was performed by centrifugation of the blood at 600 g for 20 min after it was layered atop an equal volume of Ficoll-400 (5.7 g) and sodium diatrizoate (9.0 g) per 100 ml of water (Ficoll-Hypaque). The recovered mononuclear cells were washed in alpha medium (13) minus nucleosides ($\alpha-$) to which 5% fetal calf serum was added.

The whole population of mononuclear cells was separated at room temperature into Ig-positive and Ig-negative subpopulations by Sephadex G-200 anti-human Fab column immunoabsorbent chromatography as previously described (14). Each cell population was contaminated with the other to an extent of less than 2%. The nonimmunoglobulin-bearing (Ig-negative) lymphocyte population was further fractionated into E-rosette-forming cells over a Ficoll-Hypaque density gradient (15). The E-rosette-depleted subpopulation (null cells) usually contained less than 2% Ig-positive cells, less than 10% E-rosette-positive cells, and varied between 30 and 60% EAC-positive cells. These latter cells are additionally heterogeneous and may contain pre B cells as well as monocytes. Highly purified T cells (92% E-positive) were prepared by ammonium chloride lysis of the pelleted E-rosetting cells. All cell populations were washed four times and resuspended in $\alpha-$ medium with 2% fetal calf serum. All cell population were washed four times and resuspended in $\alpha-$ medium with 2% fetal calf serum at convenient concentrations for addition of 0.1 ml of cell suspension to 0.8 ml of the plasma clot incubation system described by McLeod and co-workers (16) as modified by Clarke and Housman (5). In several experiments, T or B cells were cocultured with null cells. The culture system contained 2 international units (IU) of erythropoietin with a sp act of approximately 50 IU per mg of protein. (Prepared by Dr. Peter Dukes and provided by Dr. Anne Ball, Blood Diseases and Resources Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Md.).

Clotting was initiated by the addition of 0.1 ml of NCTC 109 (Microbiological Associates, Bethesda, Md.) containing 1 U of grade 1 bovine thrombin (Sigma Chemical Co., St. Louis, Mo.). The 1.0 ml clotting mixture was dispensed in 0.1-ml portions into 0.2 ml microtiter culture wells (Linbro plates—Linbro Scientific Co., New Haven, Conn.) and incubated under 5% CO_2 in high humidity.

The plasma clots were incubated for up to 14 days. On day 14, the clots were fixed and stained as described by McLeod and co-workers (16). Erythroid colonies in four to six clots were counted and the results expressed as the mean and standard error of the mean of the number of erythroid colonies per 10^5 cells plated. In most of the experiments, the majority of the observed erythroid colonies had the typical appearance of large BFU-E as described by Clarke and Housman (5). In certain experiments, however, particularly those in which null cell preparations were cultured in the absence of added T cells, most of the erythroid colonies were either single or contained less

than three subcolonies. Therefore, total erythroid colonies and typical large BFU-E colonies were separately counted, but both types of colonies were considered BFU-E derived because they were obtained from peripheral blood and were present in 14-day cultures. When varied numbers of T or B cells were added to null cells in the system, the results were expressed as the number of BFU-E colonies per 10^5 null cells. Throughout the experiments the standard errors of the means ranged between 4 and 15% of the mean BFU-E colony scores. Photographs were taken of the entire dehydrated fixed and stained plasma clots or of representative colonies within the clots.

Results

The Morphology of Erythroid Colonies.   The erythroid colonies that emerged in erythropoietin-dependent plasma clot cultures of unfractionated peripheral blood mononuclear cells had the characteristic morphology of BFU-E colonies (5). At 14 days, such colonies contained more than 3 (usually 10 or more) subcolonies of well hemoglobinized nucleated erythroid cells (designated as 3–4+ BFU-E colonies). However, when separated null cells were so cultured, the characteristics of erythroid colony growth were quite different. Most of the colonies were either single or contained only three or less unevenly hemoglobinized subcolonies (designated as 1–2+ BFU-E colonies). When T cells were added to null cells, the erythroid colonies progressively acquired the proliferative thrust of the BFU-E in unfractionated mononuclear cells and hemoglobinization of the subcolonies approached completion. Examples of the most frequently observed erythroid colonies that emerged in null cell cultures to which zero and increasing numbers of T cells were added are shown in Fig. 1.

Enumeration of Colonies.   Table I shows the results of five experiments in which unfractionated and fractionated mononuclear cells of normal individuals were incubated at various cell concentrations for 14 days in the presence of erythropoietin as described. The data show that BFU-E colonies were evident in cultures of unfractionated cells to the same extent as previously described (5). They were also present in the Ig-negative fractions, but recovery was irregular. BFU-E colonies of any size did not appear in cultures of Ig-positive (B cell) or T-cell populations. Well developed BFU-E colonies were observed to a variable extent in the isolated null cell fractions. In two studies, the cultures were nearly devoid of large (3–4+) BFU-E colonies but such colonies were present in three other studies. Furthermore, numerous small erythroid colonies (1–2+ BFU-E) were present in four of the five null cell preparations. These data are shown in Fig. 2 in which the number and quality of the BFU-E colonies in null cells are compared to the colonies in unfractionated cells. The total number of BFU-E colonies per 10^5 null cells was often greater than the BFU-E colonies per 10^5 unfractionated mononuclear cells, but small BFU-E colonies predominated in the null cell fractions whereas large BFU-E colonies constituted all of the erythroid growth in unfractionated mononuclear cell cultures. Photomicrographs of the general extent of erythroid colony growth in representative unfractionated, null, T-, and B-cell fractions are shown in Fig. 3 A. The more detailed morphology of erythroid colonies that vary in size and number of subcolonies is shown in Fig. 1.

Results of Mixtures of B and T Cells with Null Cells.   The data presented above suggested to us that BFU-E reside in the null cell fraction, but stimulation of their proliferation and differentiation requires T cells in vitro. The low BFU-E colony yield in the Ig-negative fractions was due, we thought, to the
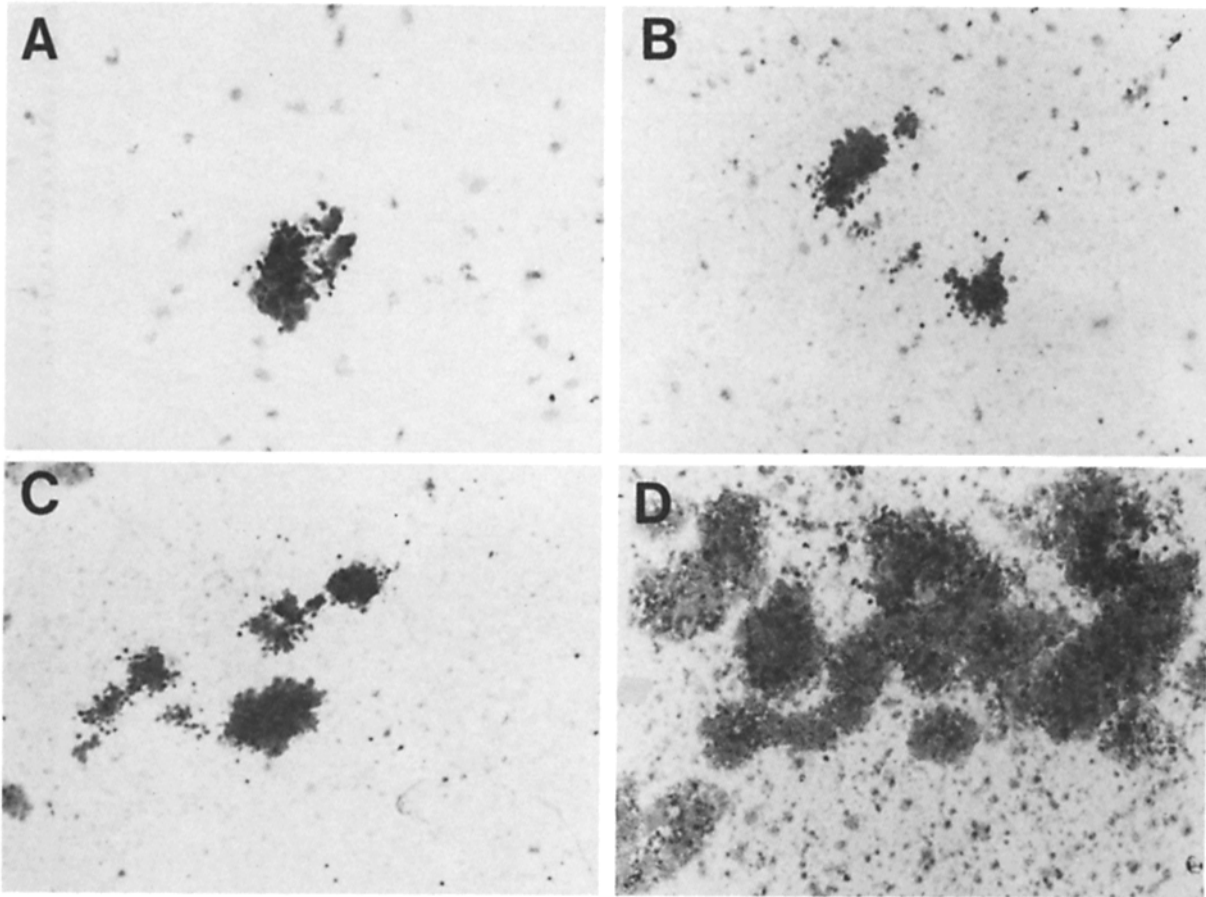
Fig. 1. The effects of serial additions of T cells on BFU-E subcolony size and number. Null cells were cultured at 10^6 per ml of clot in the presence of 2 IU of erythropoietin per ml with the addition of T cells as follows.

Panel	T cells added/ml	Symbol	Description
A	none	+	Single 8–20 cell "CFU-E-like" colony with detectable but variable hemoglobinization;
B	5×10^4	++	Cluster of three 8–20 cell colonies with detectable but variable hemoglobinization;
C	10^6	+++	Small BFU-E colony with three to five subcolonies each larger than 20 cells with variable but definite hemoglobinization;
D	5×10^6	++++	Typical BFU-E colony with multiple subcolonies of greater than 20 cells, fully hemoglobinized.

low percentage of null cells in this fraction. Conversely, the well developed BFU-E observed in certain null cell preparations could have been due to the interaction of the T cells that variably contaminate the null cell preparation. Therefore, in three experiments serial additions of either T cells or B cells were made to null cells. The total number of BFU-E colonies of all sizes per 10^5 null cells plated was determined. The results are shown in Fig. 4. In each experiment, there was striking stimulation of BFU-E colony formation by the

<div align="center">TABLE I</div>

BFU-E Colonies in Cell Fractions

Study number	Unfrac- tion- ated	Ig−		B		T		Null	
	Cell type								
	Number of cells plated per ml of clot								
	3×10^6	10^6	5×10^6	10^6	5×10^6	10^6	5×10^6	10^6	5×10^6
	BFU-E colonies per 10^5 cells plated								
1	21	2	15	0	0	0	1	7	20
2	9	0	8	0	0	0	0	0	1
3	7	3	7	0	0	0	0	0	1
4	23	ND*	14	0	0	0	0	8	1
5	16	ND	3	0	0	0	0	15	23

BFU-E colony formation in unfractionated and fractionated peripheral blood mononuclear cells from five normal individuals. The cells were plated at the indicated concentrations per ml of clot. The number of BFU-E of a size considered 3+ to 4+ as defined in the legend to Fig. 1 are included in the score. The standard errors were less than 10% of the mean in all cases.
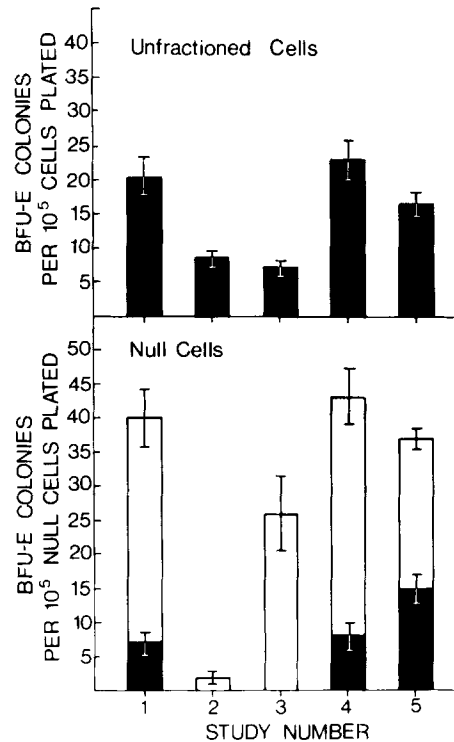
* ND, not done.

FIG. 2. Total BFU-E and 3-4+ BFU-E colonies in cultures of (top) unfractionated mononuclear cells and (bottom) null cell fractions derived from the same samples. The height of each bar represents the total number of BFU-E colonies regardless of size. The solid areas represent the proportion of 3-4+ BFU-E colonies. The data are expressed as the number of observed colonies per 10^5 cells plated.

addition of T cells but not by the addition of equal numbers of B cells. The different effects of B and T cells at 5×10^6 per ml of clot on the number of BFU-E colonies formed by null cells at 10^6 per ml of clot is also illustrated by the photomicrographs in Fig. 3 B. The colonies that occurred after B-cell addition were nearly all granulocyte colonies. These are compared to erythroid colonies in Fig. 5. In addition to the effects of T cells on the *number* of BFU-E colonies in T:null cell mixtures, there was also a marked effect of T cells on the morphology of the induced colonies. The subcolonies in the individual BFU-E colonies increased in number and size as the T-cell concentration was increased. This effect is illustrated in the photomicrographs in Fig. 1.

Effects of Different T-Cell Preparations on BFU-E Formation. Having established that fresh isogeneic T cells enhance the proliferation and differentiation of the BFU-E in null cells, the influences of gamma irradiation (3,000 rads in 2 min), removal of adherent cells and allogeneic effects were next investigated. Separated null cells at 10^6 per ml of clot were either cultured alone or cocultured with fresh isogeneic T cells, irradiated T cells, nylon filtered T cells, or allogeneic T cells. In one study, irradiated null cells were cocultured with 5 $\times$ 10^6 nonirradiated isogeneic T cells. The results are shown in Fig. 6. Allogeneic and nylon filtered T cells were as effective as fresh isogeneic T cells in the induction of large BFU-E colony formation. In a separate experiment (data not shown), null cells were nylon filtered and cultured at 10^6 cells per ml with 5×10^6 T cells. The removal of adherent cells did not reduce the effect of T cells on BFU-E colony formation by null cells. In contrast, irradiated T cells did not enhance BFU-E colony formation and prior irradiation of the null cells rendered them completely incapable of any erythroid colony formation even when fresh isogeneic T cells were present (Fig. 6).

Effects of T-Cell Conditioned Medium. The experiments described above suggested that T cells capable of DNA synthesis must render the BFU-E in the null cell population capable of proliferation and differentiation in vitro. Removal of adherent cells from the T or null cell fractions did not influence this phenomenon. B cells were ineffective. To determine whether a soluble factor released by dividing T cells might induce BFU-E colony formation in null cells, we studied the effects of a conditioned medium in which T cells had been stimulated to divide by tetanus toxoid (17, 18).[3] This conditioned medium contains a factor(s) that is known to stimulate the proliferation and differentiation of T and B cells. The factor(s) has been called lymphocyte mitogenic factor. The conditioned medium was prepared exactly as described by Geha and his co-workers (17) except that NCTC 109 was utilized in the preparative procedure instead of medium RPMI 1640. Addition of this conditioned medium to null cells was made by substituting it for NCTC 109 in the plasma clot system up to 20% of the total volume of the clot. Four such studies were performed. Representative examples of the data are shown in Figs. 3 B and 7. Erythroid colonies particularly large BFU-E colonies were clearly induced to proliferate and differentiate by T-cell conditioned medium. In fact the medium was as potent as 2.5×10^6 fresh T cells per ml. Tetanus toxoid had no direct effect on BFU-E colony formation. In addition, an extract of human fetal

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[3] R. S. Geha, F. Mudawwar, and E. Schneeberger. 1977. The specificity of T-cell helper factor in man. *J. Exp. Med.* **145:** 1436.
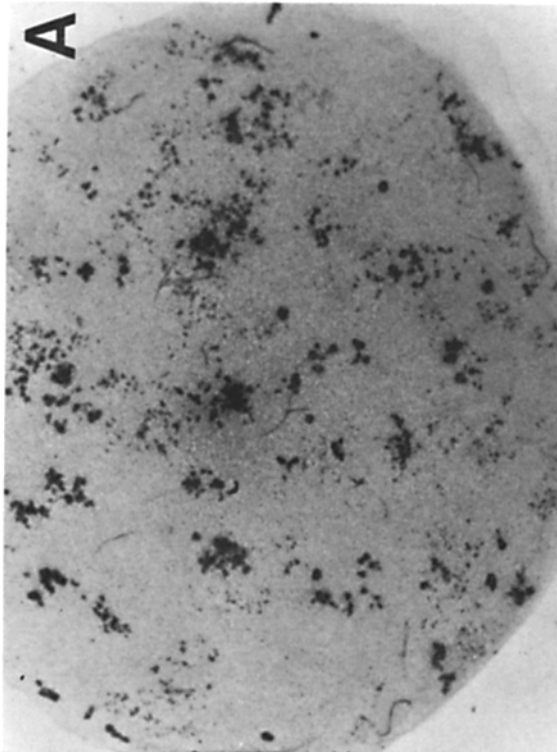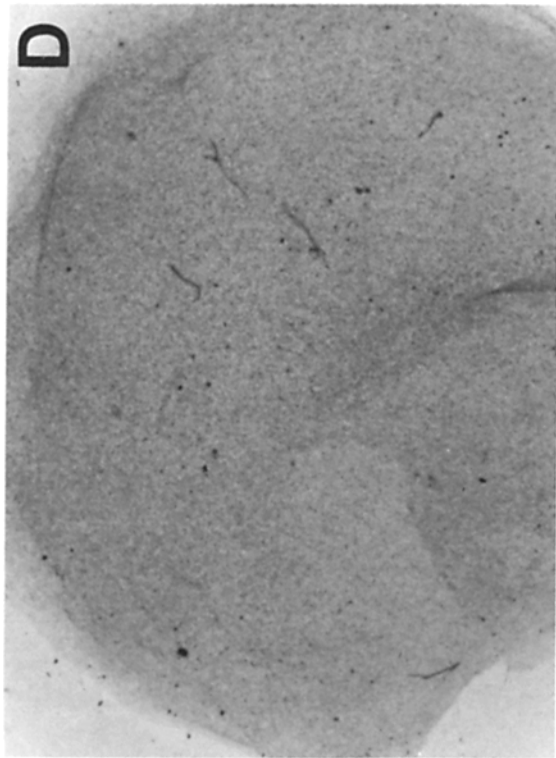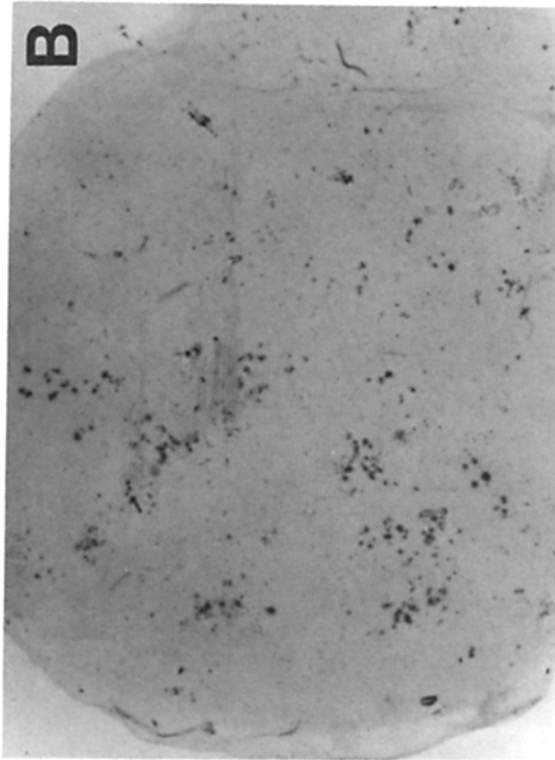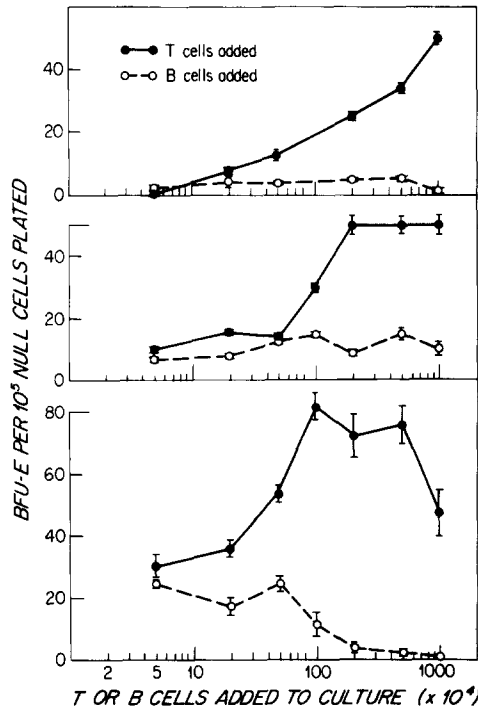
FIG. 4. Three examples of the effects of varying numbers of isogeneic T (●) and B (O) cells on the formation of BFU-E colonies. The T or B cells were added to 10^6 null cells per ml of plasma clot in the presence of 2 IU of erythropoietin at the cell concentrations shown in the abscissa. The ordinate represents the total number of BFU-E colonies of all sizes observed after 14 days of culture of the cell mixtures. In addition to the numerical increase in BFU-E colonies observed when T cells were added to null cells, there was also an increase in the average size of individual colonies. The effect of T cells on BFU-E colony size is illustrated in Fig. 1.

kidney that is regularly utilized as a source of human granulocyte colony-stimulating activity (CSA) was also examined in this system. This preparation of CSA had no such stimulating effect (data not shown). Finally, the effects of the T-cell conditioned medium on a CSA-dependent human granulocyte colony-forming system (19) was evaluated. The medium did not provide granulocyte

FIG. 3A. Photomicrographs of fixed and stained 0.1-ml plasma clots of various preparations of blood mononuclear cells after 14 days of culture. The darkly stained colonies are benzidine positive.

Panel	Description
A	3×10^6/ml unfractionated cells
B	10^6/ml null cells
C	5×10^6/ml T cells
D	5×10^6/ml B cells

Note the extensive growth of BFU-E colonies in panel A and the small "CFU-E-like" colonies in panel B, the null cell fraction. Some large (3–4+) BFU-E colonies are also present in the null cell fraction.
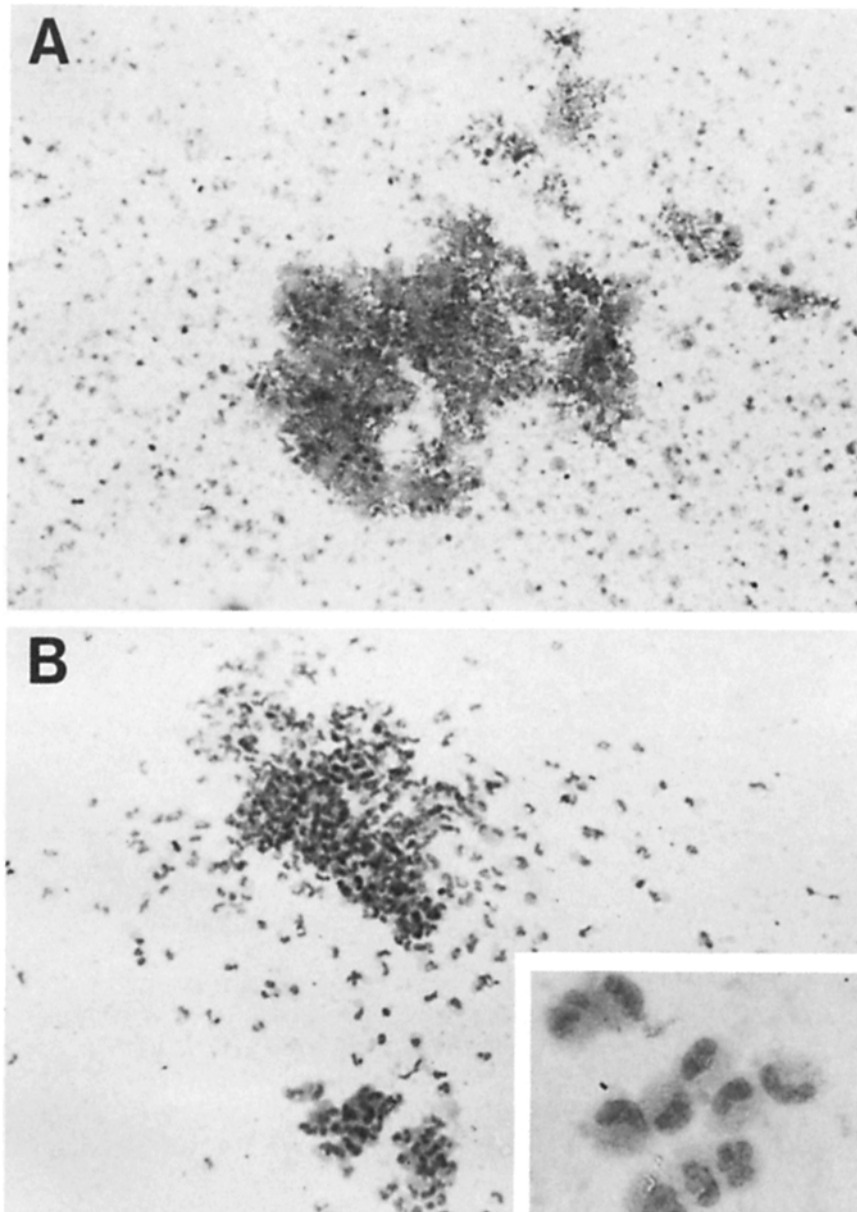
FIG. 5.   Typical granulocyte colony induced by addition of 5×10^6 B cells to 10^6 null cells (panel B). The insert provides a better definition of the granulocyte morphology. The colony is compared to a typical BFU-E colony induced by 5×10^6 T cells (panel A).

colony-stimulating activity in these experiments in which serial dilutions of the conditioned medium up to 20% vol/vol did not substitute for a human macrophage feeder layer in granulocyte colony formation. (Data kindly provided by Dr. Peter Quesenberry, Peter Bent Brigham Hospital, Boston, Mass.).
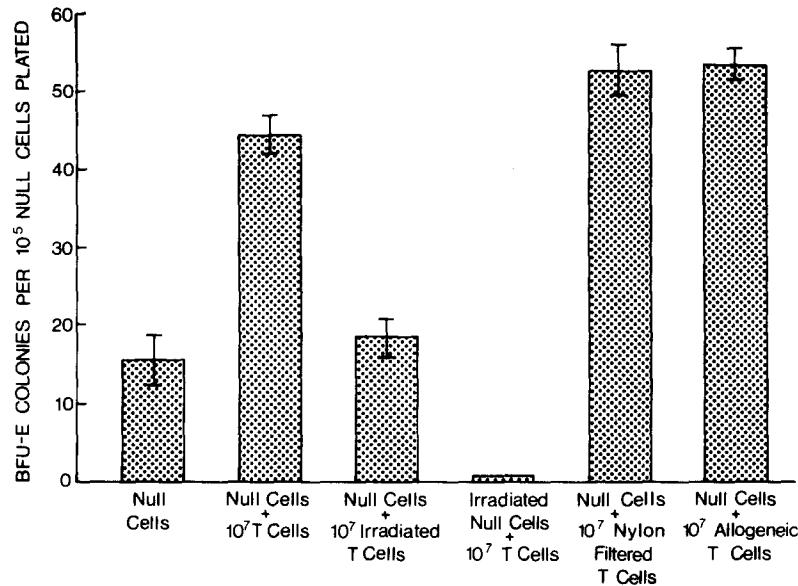
FIG. 6. Influence of various T-cell preparations and the effects of irradiation on 3-4+ BFU-E colony growth in null cells. The fresh or irradiated null cells were incubated at 10^6 cells per ml, and 10^7 variously treated isogeneic or allogeneic T cells were added to them. The cell mixtures were incubated for 14 days in the presence of 2 IU of erythropoietin. Only 3-4+ BFU-E colonies were included in the colony scores.

Discussion

The hematopoietic precursor cells that are contained among peripheral blood mononuclear cells include totipotential hematopoietic stem cells (20) and committed granulocyte (21) and erythroid precursors (5, 12).[2] Recent studies of human marrow and peripheral blood in culture show that approximately 1% of the earliest recognizable erythroid precursors, the BFU-E may be found in peripheral blood.[2] These cells require the addition of erythropoietin to induce their proliferation and differentiation.

In previous studies of separated human null, T and B cells, it was reported that the erythroid and granulocyte committed stem cell populations reside in the null cell fraction (21, 22). The data presented in Table I support that impression with respect to the erythroid precursors. However, the growth characteristics of erythroid colonies observed when null cells are cultured with erythropoietin in the absence of added T cells indicate that the proliferative capacity and extent of differentiation of blood BFU-E are severely curtailed in this setting. Under these conditions, most of the erythroid colonies are small and single. Only when T cells are added to null cells are typical BFU-E colonies regularly observed in large numbers, and this effect of T cells is concentration dependent. Neither T nor B cells alone induce any erythroid colonies at any cell concentration tested, but as shown in Figs. 1, 3 B and 4 the addition of T cells to null cell cultures dramatically influences the number and quality of BFU-E colonies derived from a given number of null cells. B cells do not share this helper function.
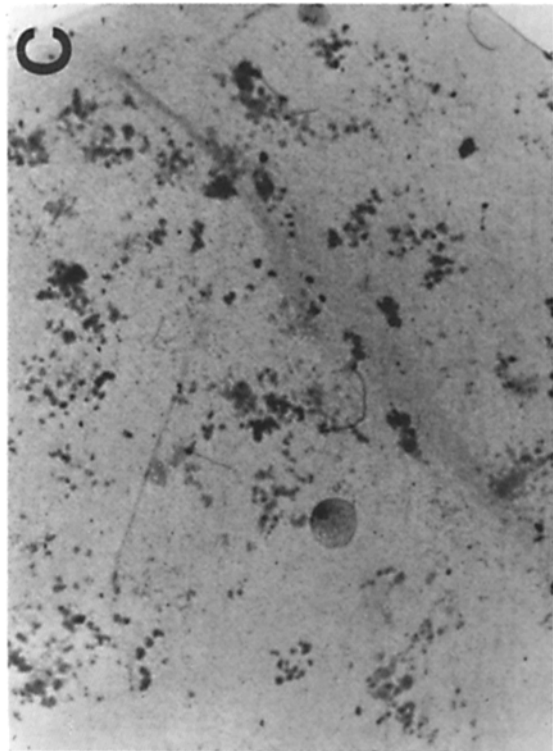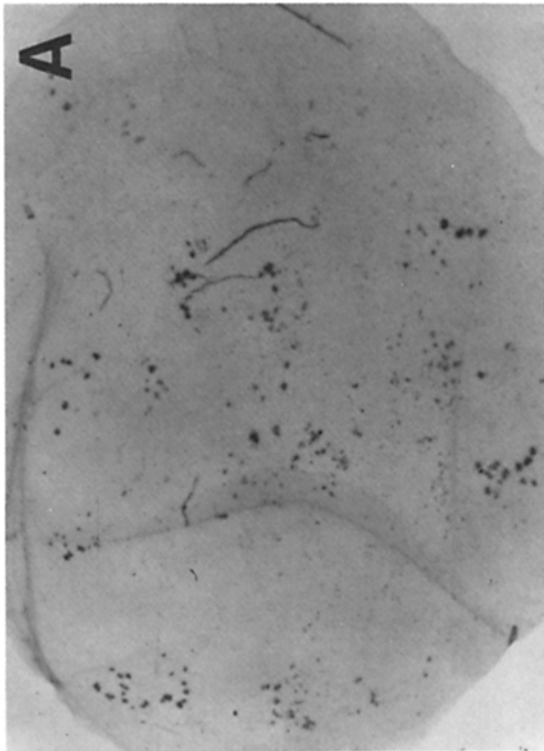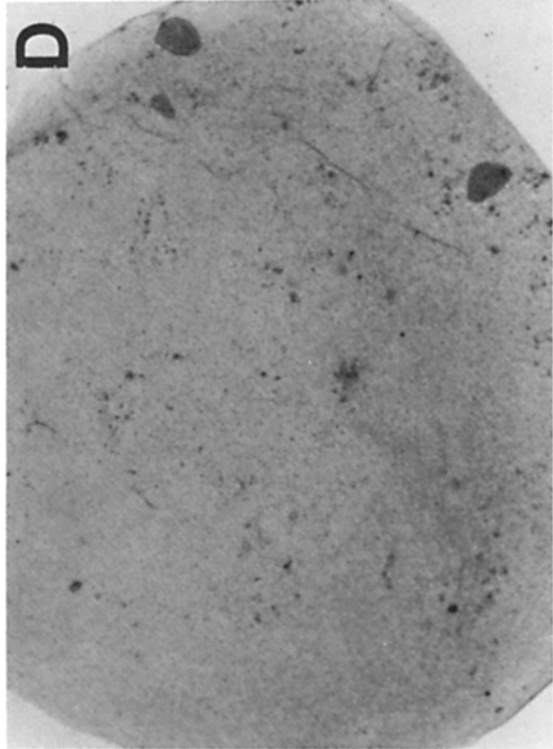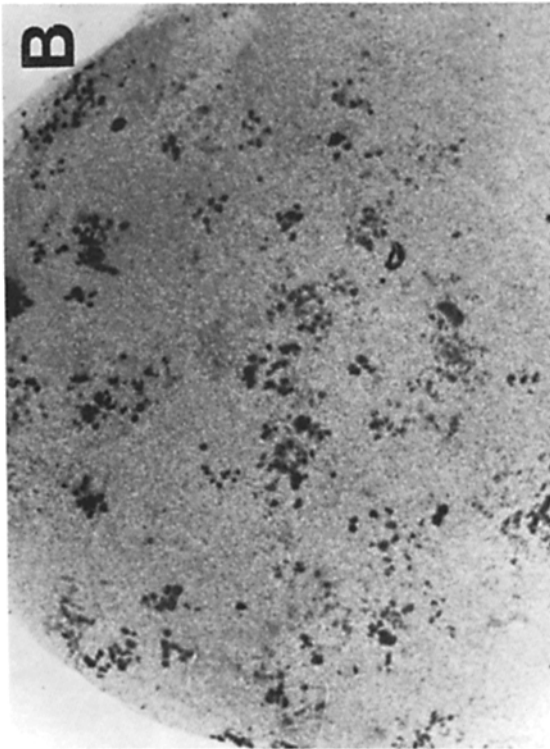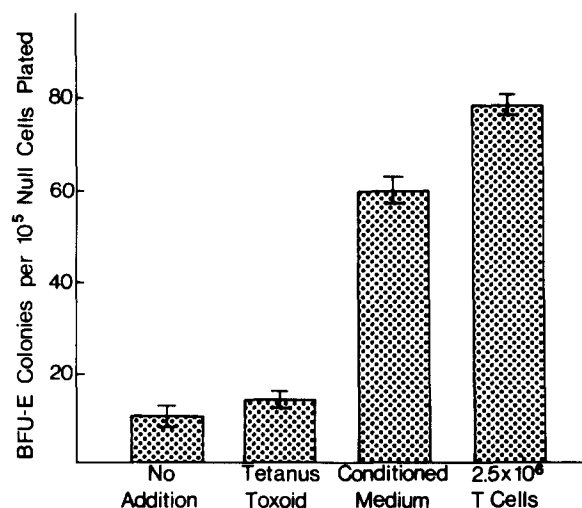
FIG. 7. Influence of T-cell conditioned medium and intact T cells on 3-4+ BFU-E colony growth in null cells. The null cells were incubated at 10^6 cells per ml of clot in the presence of 2 IU erythropoietin. The different additives were tetanus toxoid, at a final concentration of 4 μg per ml of clot, T-cell conditioned medium, prepared as described in the text, at 20% vol/vol or 2.5 × 10^6 T cells per ml of clot. The mixtures were cultured for 14 days and the 3-4+ BFU-E colonies were enumerated.

That T cells provide a helper function in antibody synthesis by B cells is well established. Recent experiments have also provided evidence that lymphocyte populations influence the production of eosinophils in vivo (23-26). Schimpl and Wecker (27) recognized that helper T-cell function could be replaced by soluble products elaborated by T cells in culture. Such factors have been explored in some depth by Geha and his co-workers (17, 18).[3] In this report we demonstrate that a soluble factor elaborated by antigen-stimulated T cells in culture induces erythropoietin-dependent BFU-E colony growth in a null cell preparation that exhibits minimal erythroid colony growth in response to erythropoietin alone. Johnson and Metcalf (27 A) have shown that pokeweed mitogen-stimulated murine spleen-conditioned medium markedly enhances erythroid colony formation in cultures of murine liver cells. In fact they found that such a conditioned medium may substitute for the addition of erythropoietin to the culture system. That lymphocytes may produce granulocyte colony stimulation factor under certain circumstances is also well established (27B, 27C).

FIG. 3B. See legend to Fig. 3 A.

Panel

A	10^6/ml null cells
B	10^6/ml null cells + 5 × 10^6/ml T cells
C	10^6/ml null cells + T-cell conditioned medium
D	10^6/ml null cells + 5 × 10^6/ml B cells

Note extensive growth of BFU-E colonies in null cell cultures to which either T cells or T-cell conditioned medium is added. The two large grey areas on the right-hand side of panel D are artefacts and the colony in the center is a granulocyte colony.

Neither the mechanism nor the specificity of the erythropoietic-inducing product(s) elaborated by T cells is defined in this initial report. Possibly the elaborated factor(s) somehow render putative erythropoietin receptors on committed stem cell surfaces more accessible to erythropoietin. At the concentrations explored here, this T-cell conditioned medium appeared relatively specific. It did not support granulocyte colony growth, but other T-cell mitogens might produce different products that may stimulate both erythroid and granulocyte colony growth.

The significance of these in vitro findings with respect to erythropoiesis in vivo is presently unknown. Certain data suggest that thymocytes may influence murine hematopoiesis in vivo (28–30). In fact, it has recently been shown that an anti-theta serum blocked the reconstitution by marrow transplantation of the erythroid marrow of W/W^v anemic mice. The addition of thymocytes to anti-theta serum-treated normal marrow reduced the deleterious effects of this antiserum (31). An argument against a governing role of T cells in human erythropoiesis in vivo is posed by the fact that infants with severe combined immune deficiency (SCID) or the DiGeorge syndrome, who lack mature T cells, are not anemic unless they are infected or have graft versus host disease. However, recent studies have shown that infants with SCID do have cells in their peripheral blood that are precursors to T cells (32, 33). Perhaps in such patients the precursor cells can elaborate an erythroid colony growth factor in vivo. Though in vivo significance is yet to be understood, our data demonstrate that isogeneic and allogeneic T cells influence the proliferation and differentiation of the erythroid precursors in null cells in vitro. This new finding now permits us to examine a variety of human disorders of erythroid cell production to determine whether a particular defect resides in the null or stem cell fraction or in the T or helper-suppressor fraction.

Summary

Human mononuclear leukocytes were fractionated into populations of null, T and B cells by immunoabsorbent column chromatography followed by E-rosette formation and purification of T cells by differential centrifugation and osmotic lysis. The unfractionated and fractionated cell populations were first separately cultured for 14 days in plasma clots in the presence of two international units erythropoietin. Typical erythroid burst-forming unit (BFU-E)-derived colonies grew in the unfractionated cell cultures but not from T- or B-cell cultures. BFU-E colonies grew in null cell cultures but most of the colonies were small and variably hemoglobinized with less than three subcolonies. When intact T cells were added to null cells and cocultured, many typical large BFU-E colonies with more than 10 well homogenized subcolonies appeared. Increasing numbers of large BFU-E colonies in null cell cultures were induced by stepwise addition of T cells but not by the addition of B cells. A conditioned medium in which T cells had been induced to divide by tetanus toxoid substituted for intact T cells in this T-cell-dependent BFU-E colony formation observed in null cells.

These findings demonstrate that the BFU-E, a committed erythroid stem cell, resides in the null cell fraction of peripheral blood, but its proliferative

capacity and differentiation in vitro requires a soluble product of T cells.

Such experiments now permit a new approach to the assessment of various disorders of erythropoiesis. Erythroid hypoplasia in a particular case may be due to dysfunction of the committed precursor cell or to a failure of a helper effect induced by T cells.

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