MATURE BONE MARROW ERYTHROID BURST-FORMING UNITS DO NOT REQUIRE T CELLS FOR INDUCTION OF ERYTHROPOIETIN-DEPENDENT DIFFERENTIATION*

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Studies of murine erythropoiesis in vivo (1, 2) and of human and murine marrow and blood in vitro have shown that the production of erythrocytes is regulated by an orderly process of maturation and terminal differentiation of committed erythroid progenitors presumably derived from a tripotent hematopoietic stem cell (CFU-S) (3, 4).

At least two classes of committed erythroid progenitors exist in human blood and marrow. The most mature and numerous of these, the marrow erythroid colony-forming unit (CFU-E) is an immediate progenitor of the proerythroblast (3, 5). This progenitor rapidly forms single colonies of terminally differentiated erythroid cells in vitro under the influence of relatively low concentrations of erythropoietin and is, in turn, the product of a less-mature progenitor, the erythroid burst-forming unit (BFU-E) (3, 5). The behavior of BFU-E in culture is characterized by division and migration before arrival at the CFU-E stage and terminal differentiation.

More recent studies have shown that BFU-E are themselves heterogeneous. They give rise to colonies of varying size, subcolony number, and fetal hemoglobin content (6, 7). Not surprisingly, it is believed that BFU-E that give rise to large colonies capable of gamma-chain expression are less mature than those that give rise to small colonies and predominant hemoglobin A (7). Furthermore, the process of erythropoiesis is thought to involve a programmed maturation of immature BFU-E to mature BFU-E, during which time, enhanced sensitivity of the progenitor to erythropoietin for terminal differentiation might occur (7, 8).

Though BFU-E are largely found in marrow, a small fraction of these progenitors is detectable in peripheral blood (9, 10) within the null-cell fraction (11, 12). However, these peripheral blood progenitors do not exhibit erythropoietin-dependent differen-

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Abbreviations used in this paper: a erythrocyte(s), erythrocytes that contain hemoglobin A, α-; α medium minus nucleosides, BFU-E, erythroid burst-forming unit(s), CFU-E, marrow erythroid colony-forming unit(s), CFU-S, tripotent hematopoietic stem cell(s), FACS, fluorescence-activated cell sorter, F erythrocyte(s), erythrocytes that contain hemoglobin F and A, FCS, fetal calf serum, LMF, lymphocyte mitogenic factor, SCID, severe combined immunodeficiency.
tiation into typical hemoglobinized BFU-E colonies in the absence of T cells or factor(s) derived from appropriately stimulated T cells (11). A similar inducer role of what has been called lymphocyte mitogenic factor (11), leukocyte conditioned medium (4), or burst-promoting activity (8) has been noted in several laboratories.

One might predict from such observations that a particular deficiency of T cells or T cell function in vivo might be associated with erythroid hypoplasia, but such a complication of immune deficiency has not been regularly reported and, furthermore, would only occur if T cells were additionally required to promote the erythropoietin-dependent differentiation of the mature BFU-E that numerically dominate the marrow pool of these progenitors.

To examine the latter possibility, we have utilized a complement-fixing cytolytic monoclonal antibody that reacts with all circulating T cells (13, 14) to determine whether elimination of whatever peripheral blood T cells might be present in marrow would depress erythropoietin-dependent differentiation of the BFU-E derived from that tissue. For comparison, we studied the effect of such antibody-mediated T cell destruction on the differentiation of peripheral blood BFU-E.

The results show that, in contrast to peripheral blood BFU-E, the vast proportion of erythropoietin-dependent marrow BFU-E differentiation in vitro occurs without a requirement for T cells or T cell-derived factors in the cultures. This suggests that during the process of maturation of BFU-E, there is a loss of T cell dependence for erythropoietin-dependent erythroid differentiation.

Materials and Methods

Cell Treatment and Culture. Peripheral blood and bone marrow was obtained from normal adult volunteers. Care was taken to aspirate 2 ml of marrow, the yield of which was \( 40 \times 10^6 \pm 10 \times 10^6 \) nucleated cells contaminated with \( \leq 3\% \) peripheral T cells. The clonal assays of BFU-E and CFU-E in human bone marrow and BFU-E in peripheral blood have been previously described (9, 15). Briefly, mononuclear cells were isolated by Ficoll (Pharmacia Fine Chemicals, Div of Pharmacia, Inc., Piscataway, N J.) -Hypaque (Winthrop Laboratories, New York) centrifugation. Nonadherent peripheral blood mononuclear cells that were markedly depleted of monocytes and B lymphocytes were prepared by passage over nylon wool as previously described (16). Highly purified T cells were separated by ammonium chloride lysis of pelleted E-rosetting cells.

A monoclonal cytotoxic IgG antibody reactive with all human circulating T cells (13, 14) was prepared by hybridoma techniques as recently described (17). We presently refer to this antibody as anti-T3. Its activity was established by serial dilution of the murine ascites fluid into which it was secreted. It was used in all of these studies in a dilution of 1:500 of this fluid in minimum essential medium (S-MEM; Grand Island Biological Company, Grand Island, N Y) medium with 10 mM Hepes buffer, to which 5% fetal calf serum (FCS) (Flow Laboratories, Inc., Rockville, Md.) was added. A similarly diluted murine ascites fluid lacking monoclonal antibody activity was used as a control. Analysis of mononuclear cell preparations for the enumeration of T cells was performed with a fluorescence-activated cell sorter (FACS) (FACS II, Becton Dickinson FACS Systems, Mountain View, Calif.) (18), using anti-T3 and a fluorescein-conjugated anti-mouse IgG.

Treatments of cells with anti-T3 or the control ascites fluid were accomplished by incubation of \( 20 \times 10^6 \) to \( 40 \times 10^6 \) mononuclear cells in 1 ml of the medium in which either antibody-containing, or control, ascites fluid had been diluted at 4°C for 30 min. Then, 0.15 ml of freshly frozen rabbit serum (lot C192108, Grand Island Biological Company) was added as a source of complement. The incubation was continued at 37°C for 1 h with gentle shaking. After the incubation at 37°C, the cells were washed three times in \( \alpha \)-medium minus nucleosides (\( \alpha^- \)) (Grand Island Biological Company) plus 5% FCS. The appropriate cell numbers were added in 0.1 ml of \( \alpha^- \) plus 5% FCS to 0.8 ml of the erythropoietin-dependent
plasma clot system used by McLeod et al. (15), as modified by Clarke and Housman (9). There were two sources of erythropoietin concentrate. One was a human urinary preparation with a 28.3 IU/mg protein sp act (prepared by Dr. Peter Dukes and provided by Dr. Anne Ball, Division of Blood Diseases and Resources, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Md.). The other was prepared from the plasma of phenylhydrazine-treated anemic sheep (Connaught Step III; Connaught Medical Research Laboratory, Willowdale, Ontario, Canada), with an ~5 IU/mg sp act. Both were used at a final concentration in the plasma clot of 2 IU/ml. In some experiments, 0.1 ml of medium in which tetanus toxoid-stimulated mononuclear cells had proliferated was substituted for that volume of NCTC-109 (Microbiological Associates, Bethesda, Md.). This medium contains lymphocyte mitogenic factor (LMF) (11) activity. Clotting was initiated by the addition of 0.1 ml of NCTC-109 that contained 1 U of grade I bovine thrombin (Sigma Chemical Company, St. Louis, Mo.). 0.1 ml aliquots of the clotting mixture were then dispersed in 0.1-microliter culture wells (Linbro Chemical Co., Hamden, Conn.) and incubated in 5% CO2 in high humidity. CFU-E were enumerated on day 7 and BFU-E on day 11-14.

Data Presentation. All of the data are expressed as the number of colonies/10^6 non-T cells plated. This became important only in analyses of peripheral blood in which substantial numbers of T cells were removed by treatment with anti-T3, but a constant number of cells was maintained in the plasma clot. The quantitation of the non-T cell content of mononuclear cells was made from the FACS analysis.

Classification of BFU-E. All erythroid colonies with three or more subcolonies were included in the total BFU-E count, but a semiquantitative analysis of the size of BFU-E was made from morphologic inspection, and the grading system of average BFU-E size previously described (11) was employed to define the appearance of the colonies.

Results

FACS Analysis of Peripheral Blood and Marrow Mononuclear Cells. Fig. 1A shows a representative FACS analysis of Ficoll-Hypaque-separated nonadherent peripheral blood mononuclear cells. In this study, 77% of the mononuclear cells were identified as mature T cells by indirect immunofluorescence with anti-T3. Fig. 1B shows the FACS analysis of this population after treatment with anti-T3 and complement. It is readily demonstrated that the mature T cells which comprised the majority of peripheral blood lymphocytes were virtually eliminated by this treatment. FACS analysis of bone marrow mononuclear cells was always quite different. As shown in Fig. 2, a representative analysis, there were virtually no mature T cells detectable by this method in a Ficoll-Hypaque-separated sample of marrow mononuclear cells.

Effect of Treatment of Peripheral Blood Mononuclear Cells with Anti-T3 upon BFU-E Colony Expression. The FACS analysis of peripheral blood (Fig. 1A B) demonstrated the virtual elimination of mature T cells from the peripheral blood mononuclear cell population by the antibody and complement treatment. The effect of this depletion on BFU-E colony formation was then studied in two different types of experiments, examples of which are shown in Fig. 3. In the experiment shown in Fig. 3A, the control cells (treated with mouse ascites fluid and complement) were cultured alone and in the presence of varying proportions of LMF in the plasma clot system. BFU-E colony formation was not affected morphologically or numerically by this treatment, even in the presence of added LMF. In contrast, treatment of the cells with anti-T3 and complement greatly reduced colony formation, both numerically and with respect to colony size, unless the cultures were fortified with as little as 1% of the medium that contained LMF activity.

The experiment shown in Fig. 3B was performed differently. Control cells treated with mouse ascites fluid and complement developed BFU-E colonies in culture, and
A negative control fluorescence intensity ~ FLUORESCENCE INTENSITY $F_m$ 1. (A) FACS analysis of mature T cell content of Ficoll-Hypaque-separated nonadherent peripheral blood mononuclear cells. Analysis was done using anti-T3 with a fluorescein-conjugated anti-mouse IgG. The area under the curve shows that 77% of the cells were mature T lymphocytes. (B) A similar FACS analysis of mature T cell content of Ficoll-Hypaque-separated nonadherent peripheral blood mononuclear cells after treatment with anti-T3 and rabbit complement. There are virtually no T cells remaining in this preparation.

Fig. 1. FACS analysis of mature T cell content of Ficoll-Hypaque-separated bone marrow mononuclear cells. There are virtually no T cells in this preparation.
growth was enhanced in this experiment by added T cells or LMF, suggesting that this sample differed somewhat from the one obtained for the experiment shown in Fig. 3A in that the inducer substance(s) released by T cells, though present in this culture, was not at an optimal level. Treatment of the cells with anti-T3 and complement greatly reduced BFU-E colony formation, both numerically and morphologically, and growth was restored by added T cells and by LMF-containing
medium when it was present at 10% of the plasma clot. T cell preparations themselves exhibited virtually no growth of BFU-E colonies, indicating minimal BFU-E contamination.

Effect of Treatment of Bone Marrow Mononuclear Cells with Anti-T3 and Complement upon BFU-E and CFU-E Colony Expression. A representative example of such a study is shown in Fig. 4, and the results of several variations of such experiments, including the representative experiment, are shown in Table I. Fig. 4 demonstrates that treatment with anti-T3 and complement does not reduce marrow CFU-E or BFU-E colony growth. Not shown is that colony morphology was not affected, nor was the addition of T cells or 10% LMF influential after antibody and complement treatment.

![Fig 4 Influence of anti-T3 and complement (Ab + C') treatment of Ficoll-Hypaque-separated bone marrow mononuclear cells on CFU-E and BFU-E colony expression. The different additives were LMF at 10% vol/vol and 10^6 T cells/ml plasma clot culture to control and to anti-T3 + complement-treated cells.](image)

| Table I |
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**Influence of Anti-T Cell Antibody and Complement Treatment on Bone Marrow Erythroid Progenitor Colony Expression**

| Experiment 1 | Experiment 2 | Experiment 3 | Experiment 4 |
| --- | --- | --- | --- |
| BFU-E/10^3 cells plated | BFU-E/10^3 cells plated | BFU-E/10^3 cells plated | BFU-E/10^3 cells plated |
| Control | 85 ± 12.3 | 900 ± 40 | 447 ± 12.2 | 512 ± 53 |
| + LMF* | 675 ± 42 | 296 ± 105 | 493 ± 31 | 420 ± 30 |
| + T cells | 737 ± 99 | 525 ± 10 | 893 ± 50 | 455 ± 30 |

| Experiment 1 | Experiment 2 | Experiment 3 | Experiment 4 |
| --- | --- | --- | --- |
| BFU-E/10^3 cells plated | BFU-E/10^3 cells plated | BFU-E/10^3 cells plated | BFU-E/10^3 cells plated |
| Antibody + complement | | | |
| No additive | 90 ± 12.1 | 450 ± 50 | 57 ± 11 | 480 ± 30 |
| + LMF* | 679 ± 38 | 448 ± 45 | 388 ± 31 | 368 ± 22 |
| + T cells | 843 ± 48 | 445 ± 10 | 285 ± 65 | 480 ± 14.2 |

* LMF at 10% vol/vol plasma clot culture
† T cells at 10^6/ml plasma clot culture
§ —, not done
in the studies in which they were used. In other studies reviewed in the table, LMF or T cells, when added to control cells, were also without effect; indicating that, in our hands at least, the culture system is usually optimal with respect to whatever LMF or T cell products may be required for the expression of CFU-E and BFU-E colonies in vitro.

Discussion

The results of these studies clearly demonstrate that in vitro requirements for the terminal differentiation of mature human marrow BFU-E differ substantially from the requirements for differentiation of the peripheral blood BFU-E. The addition of T cells, or their products, is not necessary to induce marrow BFU-E differentiation, whereas one or the other is required for optimal differentiation of peripheral blood BFU-E.

The requirement for T cells or their products for peripheral blood BFU-E differentiation (11) was previously examined in studies in which the progenitor-rich mononuclear cell population was physically separated from accompanying B and T lymphocytes (19). The role of T cells as inducers of erythropoietin-dependent erythroid burst formation in null cells was then observed. At approximately the same time, several laboratories reported requirements for conditioned media in which T cells had proliferated in response to mitogens for optimal human and murine marrow and blood BFU-E colony formation (4, 8, 20, 21).

One of the most interesting of these studies is that of Eaves and Eaves (4), who utilized the methylcellulose culture system to analyze the differentiation of marrow BFU-E into erythroid colonies. That culture system permits the continuous observation of the accrual of newly forming colonies, each of which require varying periods of growth to obtain maximal size and hemoglobin accumulation. Eaves and Eaves found that such mitogen-stimulated conditioned media were only required for the optimal differentiation of what appeared to be the most primitive BFU-E in marrow culture (4), progenitors that constitute only ~15% of the total marrow BFU-E population (22). These observations lead to the logical conclusion that marrow BFU-E represent a very heterogeneous population of cells of varying maturity, and that peripheral blood BFU-E might represent a relatively immature subset of the total BFU-E population. Indeed, the bulk of marrow BFU-E have been found to differ in several respects from peripheral blood BFU-E. In addition to their differences in T cell requirements for differentiation in culture, peripheral blood BFU-E represent only ~1% of the total marrow and blood BFU-E population (23). In our hands, their development into colonies requires higher concentrations of crude erythropoietin in the plasma clot culture system than does the development of the majority of marrow progenitors (24). A smaller proportion of peripheral blood BFU-E than of marrow BFU-E are in the process of DNA synthesis (25). Finally, hemoglobin F synthesis is easily demonstrable in almost all of the colonies derived from the growth and differentiation of peripheral blood BFU-E (26–28), whereas hemoglobin F synthesis is relatively lower (28) and often difficult to demonstrate (26) in plasma clot cultures derived from completely normal human subjects. All of the differences described above support the impression that peripheral blood BFU-E represent a subset of the BFU-E population that is less mature, i.e., closer in development to CFU-S, than is the bulk of the marrow BFU-E population. It is this relatively primitive progenitor
population that appears to require a product that can be derived from T cells to induce the interaction of the progenitor with erythropoietin and to permit differentiation into colonies.

It must be emphasized, however, that the studies of marrow BFU-E colony formation reported here and elsewhere provide insight only into differentiation of these BFU-E into erythroid colonies and not into their replication. The role of erythropoietin in erythroid-progenitor development has been explored by Udupa and Reissman (1). They clearly demonstrated that the replication of mature BFU-E and CFU-E is highly erythropoietin responsive, whereas the development of immature BFU-E from CFU-S and their replication is much less, if at all, responsive to the hormone. Amplification of erythropoiesis occurs as BFU-E mature and replicate in response to ambient erythropoietin.

Thus, we conclude that primitive BFU-E are initially derived from CFU-S by a stochastic maturation process. These early-committed progenitors replicate very slowly and progressively mature. During that maturation process, they develop increasing sensitivity to erythropoietin such that at ambient levels of the hormone, they replicate more rapidly and amplify the system. When maturing and replicating, BFU-E acquire still more erythropoietin sensitivity, they finally differentiate to form CFU-E and then adult erythrocytes. These adult erythrocytes contain 100% hemoglobin A (A erythrocytes) and comprise ~95% of the circulating erythrocytes in normal individuals. Mature T cells or their products are not involved in this process of differentiation. In contrast, the differentiation of primitive BFU-E into F erythrocytes (erythrocytes that contain hemoglobin F and A), a process that is enhanced during stress erythropoiesis (29), requires induction by T cells or their products as an adjunct to high levels of erythropoietin. T cells undetectable by these methods or T cell precursors may fulfill this role in vivo.

Examination of erythropoiesis in severe combined immunodeficiency (SCID) provides only a limited opportunity to evaluate the physiological relevance of this concept. Patients with SCID have prothymocytes and early thymic precursors that mature abnormally or not at all. The T cells of these patients as such do not promote lymphoid differentiation (30, 31). Because the differentiation of mature BFU-E to erythrocytes does not require T cells or their products, basal erythropoiesis should be evident in these patients. In fact, most of them are not more anemic than their burdens of infection would produce. One might expect, however, that stress erythropoiesis and the production of F erythrocytes would be limited in such patients unless the immature thymocytes present in their marrow secrete the inducer factors that act as adjuncts to immature BFU-E differentiation. Unfortunately, the age most of these patients and the concomitant expression of fetal hemoglobin synthesis interfere with the assessment in them of F erythrocyte production as an index of stress erythropoiesis.

A pressing biological question raised by these and other studies concerns the chemical basis of T cell induction of antibody synthesis (32, 33), eosinophil production (34), and T cell proliferation, to say nothing of erythroid differentiation. With respect to the erythroid system, the understanding of this molecular biology awaits purification of sufficient quantities of labeled functional erythropoietin and isolation of the progenitor cell itself so that the interaction of the hormone with its target can be studied in reasonably homogeneous systems. In recent work (35), a subset of T cells defined by a monoclonal hybridoma antibody, anti-T4, has been found to be
responsible for LMF production. This same subset of cells is required for B cell proliferation and differentiation, secretion of immunoglobulins, as well as generation of cytotoxic T cells (36–38). Further investigation into the T cell subsets responsible for induction of erythropoietin-dependent erythroid differentiation will more specifically identify the inducer cell in that system.

Summary

Cell-cell interactions between mature T cells and peripheral blood null cells induce erythropoietin-stimulated differentiation of peripheral blood-derived erythroid progenitors. By the use of a complement-fixing cytolytic murine hybridoma antibody uniquely reactive with mature T lymphocytes, this dependence of immature peripheral blood erythroid burst-forming unit (BFU-E) differentiation upon mature T cells or a T cell conditioned medium is confirmed. By using the same antibody, it is demonstrated that the differentiation of mature bone marrow BFU-E does not require either mature T cells or lymphocyte mitogenic factor. These findings do not preclude the presence in the bone marrow of other cells, perhaps even immature T cells, that influence erythropoietin-dependent erythroid differentiation of mature marrow BFU-E.

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