The Regulatory Role of Interleukin 2–Responsive T Lymphocytes on Early and Mature Erythroid Progenitor Proliferation

By Zeev Estrov, Chaim Roifman, Yao-Ping Wang, Tom Grunberger, Erwin W. Gelfand, and Melvin H. Freedman

To analyze the role of T lymphocytes in human erythropoiesis, we evaluated the effect of recombinant interleukin 2 (IL 2) on marrow CFU-E and BFU-E colony formation in vitro. IL 2 resulted in an increase in CFU-E and BFU-E colony numbers in a dose-dependent manner. This increase could be prevented by anti-Tac, a monoclonal antibody to the IL 2 receptor. Moreover, anti-Tac on its own resulted in an overall decrease in colony numbers. Depletion of narrow adherent cells did not alter the effect of either IL 2 or anti-Tac on colony growth. Following the removal of marrow T lymphocytes, CFU-E and BFU-E colony formation proceeded normally; however, the effects of IL 2 and anti-Tac were markedly diminished. Readdition of T lymphocytes to the cultures restored the IL 2 effect. Although T lymphocytes were not themselves essential for in vitro erythropoiesis, our studies suggest that IL 2 and IL 2–responsive T cells can regulate both early and mature stages of erythroid differentiation.

SINCE the development of in vitro erythroid colony assays, many conflicting observations regarding the influence of T cells on erythroid burst-forming units (BFU-E) have been reported. Some investigators found that monocytes rather than T lymphocytes are the major population of cells that produce burst-promoting activity (BPA), whereas others have shown that T lymphocytes are a potent source of BPA and have a regulatory effect on BFU-E. However, the influence of T cells on erythroid colony-forming units (CFU-E) has not been studied in depth, and it is accepted that the terminal stages of erythroid differentiation are regulated by erythropoietin. Interleukin 2 (IL 2), which is produced by T lymphocytes and is required for the proliferation of activated T cells, was found to enhance T lymphocyte production of a variety of colony-stimulating factors.

In the present study, we show the influence of IL 2 and of an antibody that blocks its T cell receptor sites on marrow erythroid colony growth in vitro. Using CFU-E and BFU-E culture assays, we demonstrate that T lymphocytes responsive to IL 2 regulate early (as expressed by BFU-E) as well as differentiated (as expressed by CFU-E) erythroid proliferation in vitro, indicating their regulatory role in erythropoiesis.

MATERIALS AND METHODS

Preparation of cells. Heparinized (10 U/mL) bone marrow (BM) was obtained from hematologically normal adult volunteers. The donors were advised of the procedure and attendant risks and gave informed consent. These studies were approved by the Human Experimentation Committee of our institution. The BM cells were layered over Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ) and centrifuged (200 g, 10°C) for 20 minutes to remove neutrophils and red cells.

Adherent cell removal. The mononuclear cells were depleted of adherent cells by incubation on plastic Petri dishes with 10% fetal calf serum (FCS) for 90 minutes at 37°C. In this modification of the technique described by Shaw et al., nonadherent cells were removed by gentle pipetting and repeated vigorous washing of the plates with medium. This procedure was repeated until no further cells adhered to the plastic Petri dishes. The nonadherent cell fraction contained 3% monocytes according to an analysis of cytocentrifuge smears counting 300 to 500 cells using Wright's stain, nonspecific (alpha naphthyl butyrate) esterase stain, and a modification of an immunochemical technique with anti–myeloid monoclonal antibody (Coulter Immunology, Hialeah, Fla) to identify monocyte-promonocyte cells on nonadherent cell smears.

Double T cell depletion. Post-Ficoll BM mononuclear cells were incubated with neuraminidase-sensitized sheep erythrocytes (SRBC) at 4°C for two hours, followed by gradient centrifugation. This procedure was repeated twice. Cells at the interface contained <0.5% T cells as assessed by AET-sensitized SRBC rosette formation and using anti-Leu-4 and anti-Leu-5 monoclonal anti–T cell antibodies (Becton Dickinson, Mountain View, Calif). T lymphocytes were obtained by recovery of rosetted cells from the pellet following lysis with Tris-buffered ammonium chloride. In the reconstitution experiments, these T lymphocytes were washed and counted, and either 5%, 25%, or 100% of the cells were added back to 1 x 10⁶ T cell–depleted BM cells.

Lymphocyte removal using CAMPATH-1. BM cells obtained following Ficoll-Paque gradient centrifugation were then washed once in phosphate-buffered saline (PBS) and treated with a monoclonal rat antihuman lymphocyte antibody (CAMPATH-1), as previously described. No detectable T lymphocytes following this treatment were found by E rosette formation or by indirect immunofluorescence with anti–Leu-1 (Becton Dickinson, Mountain View, Calif).

Interleukin 2. IL 2, derived from cDNA for the human IL 2 gene expressed in Escherichia coli (recombinant IL 2), was provided by Biogen Research Corp (Cambridge, Mass). Following reconstitution in α-medium, the recombinant IL 2 was assayed on a murine IL 2–dependent cell line to confirm its activity and was compared to IL 2 obtained from the Gibbon cell line MLA 144, which showed similar effects. Recombinant IL 2, whose stimulatory activity was blocked by anti–IL 2 antibodies, was added to cultures at concentrations of 1.0, 2.5, 5, and 10 U/mL.

Anti-Tac antibody. Monoclonal anti-Tac antibody directed against the IL 2 receptor was provided by W. Greene (National Institutes of Health, Bethesda, Md). Its effect on an IL 2–dependent T cell line was reversible after removal of the antibody and could be overcome by high concentrations of IL 2. Hybridoma supernatant tested as a control for anti-Tac was inactive in this system as well as...
in the erythroid culture assay. Anti-Tac was added at the initiation of culture in four different dilutions of ascites fluid ranging from 1:500 to 1:5,000. For control, anti-Leu-1 (Becton-Dickinson, Sunnyvale, Calif), a monoclonal pan-T antibody of identical subclass as anti-Tac (IgG1), was added to cultures in final concentrations of 0.025 to 0.5 μg/mL.

Erythroid colony culture assay. Post-Ficoll nucleated BM cells (1 x 10⁶) were cultured in 0.8% methylcellulose with α-medium, 30% FCS, and 1% bovine serum albumin at a final volume of 1 mL per culture plate. An IL 2-free (tested on a T cell line) human erythropoietin, 1.0 U/mL (British Columbia Cancer Research Institute, Vancouver, BC), was added to stimulate erythroid colony growth. Cultures were set up in duplicate or quadruplicate and maintained at 37 °C in 5% CO₂ in a humidified atmosphere. Colonies were counted in the culture plates using an inverted microscope. Culture plates were observed daily starting from day 4 of incubation. A cluster of eight or more cells showing first signs of hemoglobinization, which occurred at day 7, was defined as a CFU-E colony, and an aggregate of more than 500 hemoglobinized cells or three or more erythroid subcolonies counted at day 14 was defined as a BFU-E colony.

Statistical analysis. The probability of significant differences between colony numbers was determined by Student’s t test.

RESULTS

Effects of the addition of IL 2 and anti-Tac on the growth of CFU-E and BFU-E. Figures 1 and 2 illustrate the results of five separate experiments from different donors in which cells were cultured in the presence or absence of either IL 2 or anti-Tac. The stimulatory effect of IL 2 on CFU-E and BFU-E and the inhibitory effect of anti-Tac were evident in a dose-dependent manner in all experiments. The maximum colony increase comparing cultures without IL 2 and those with 10 U/mL IL 2 was 38 CFU-E and 10 BFU-E/10⁵ cells plated. However, the IL 2-induced increments on CFU-E and BFU-E colony numbers with 10 U/mL and the inhibitory effects of anti-Tac with a 1:500 dilution were both significant (P < .03 and P < .05, respectively). The addition of 10 U/mL IL 2 and a 1:500 dilution of anti-Tac simultaneously to cultures did not produce a significant change compared with the control (109 ± 6 v 103 ± 4 CFU-E and 36 ± 3 v 34 ± 2 BFU-E), suggesting a negation of their respective effects and ruling out a nonspecific toxic effect of anti-Tac. In the control cultures, anti-Leu-1, the monoclonal pan-T antibody of identical subclass as anti-Tac, had no effect on CFU-E and BFU-E colony growth.

Effects of IL 2 and anti-Tac on lymphocyte-depleted BM (using CAMPATH-1) in the presence and absence of adherent cells. In Fig 3, the combined results of two separate experiments using BM from donors not used in Figs 1 and 2 are presented. The removal of adherent cells (column 3 of the Fig) did not alter the effect of IL 2 or anti-Tac on colony growth. The stimulatory effects of IL 2 on CFU-E and BFU-E colony numbers and the inhibitory effects of anti-Tac were both significant (P < .05) and were more evident than the results of the initial experiments shown in Figs 1 and 2. The removal of T lymphocytes from marrow cells by CAMPATH-1 negated the effects of IL 2 and anti-Tac in the presence or absence of adherent cells (columns 2 and 4 of the Fig).

Effects of IL 2 and anti-Tac on cultures of T lymphocyte-depleted BM and the addition of autologous T lymphocytes. Using marrow from which T lymphocytes were depleted by double E rosette depletion, neither the addition of IL 2 nor anti-Tac had any effect on colony numbers in the
CFU-E or BFU-E culture assay (columns 1 and 2 of Table 1). No change in colony numbers was observed on adding back 5% of the removed T lymphocytes to the cultures (data not presented). However, on adding 25% of the removed T cells to $1 \times 10^5$ T-depleted BM cells, IL 2 produced a colony increase of 28% CFU-E ($P < .02$) and 50% BFU-E ($P < .03$); anti-Tac resulted in a 24% reduction of CFU-E ($P < .02$) and 18% BFU-E ($P < .03$). Similar results were demonstrated on adding back 100% of the removed T cells. As a control, T cells were plated for erythroid colony growth, and no BFU-E or CFU-E colonies were identified with or without IL 2 or anti-Tac.

**DISCUSSION**

The data reported in the present study clarify some aspects of the regulatory role of T cells on the control of erythropoiesis. We examined the effect of marrow T cells on erythroid colony growth from eight marrow samples. The studies were performed on post-Ficoll mononuclear cell suspensions as well as on cell preparations following adherent cell removal, a treatment with a monoclonal antilymphocyte antibody (CAMPATH-1) that depleted B as well as T cells, and after double E rosette depletion. Both methods for T cell depletion were highly efficient and comparable. IL 2 was used in the study as an enhancer of T lymphocyte function, whereas the monoclonal antibody, anti-Tac, was used to block the effect of IL 2. The anti-Tac effect was a direct one on erythroid progenitors but was likely mediated through T cells. The adherent cell fraction played a minor role since their removal did not alter the findings.

Although Zuckerman reported that human BFU-E growth in culture was dependent on the presence of monocytes but not T lymphocytes, our findings that adherent cells are not essential for erythropoiesis are in agreement with other laboratories. Moreover, we have convincingly confirmed that BFU-E growth in culture, although not dependent on T cells, is enhanced by T cells, as originally suggested by Mangan et al.

An analysis of the published studies dealing with nonhematopoietic cellular regulation of erythropoiesis reveals several technologic problems that could account for the contradictory reports that have emerged. First, marrow cell populations are highly heterogenous with varying numbers of subpopulations from sample to sample, even after Ficoll or other cell separation procedures. The interaction of the subpopulations cannot be quantitated or controlled and could make a comparison of various studies difficult and confusing.

Second, the methods for removal of specific cell fractions are imperfect and do not always yield a complete depletion of the cell population of interest. A small number of residual cells could influence erythroid colony growth in vitro and lead to erroneous conclusions. In addition, the depletion techniques may not remove progenitors that in turn could differentiate in culture and ultimately affect erythroid colony growth as previously suggested or secrete interleukin 1 (a macrophage product that is probably necessary for IL 2 production). With respect to the T cell depletion techniques that we used, detailed studies by others using various techniques to detect residual T cells after rosetting or CAMPATH-1 cytolysis have shown that no completely effective method is available. Similarly, with the adherence method to remove monocytes and macrophages we expect some residual cells in the depleted fraction. Another factor that must be taken into account in the interpretation of data is the enrichment effect on CFU- and BFU-E when T cells
and/or adherent cells are removed, but when the same number of cells are plated in all the studies.

In our experiments, we attempted to circumvent many of these problems by using recombinant IL 2, a specific stimulator of activated T cells, and the monoclonal anti-Tac antibody, a blocker of IL 2 receptor sites. These two reagents afforded an opportunity to explore the interaction of erythroid progenitors and T cells in a functional way. Another novel approach was to deplete T cells using the monoclonal antibody CAMPATH-1 in addition to the standard E rosette depletion technique. The T cell depletion studies confirmed that CFU-E and BFU-E growth was T cell independent, and the CAMPATH-1 studies indicated that CFU-E and BFU-E growth was probably B cell independent as well since the antibody eliminates both T and B cells.17

According to previous studies, T lymphocytes can regulate BFU-E growth primarily by producing BPA.13,27 Our findings indicate that the erythropoietic regulatory role of the T lymphocytes can be mediated by IL 2 and affect CFU-E as well as BFU-E. We have concluded that T lymphocytes are not essential for in vitro erythropoiesis; however, T cells do have a regulatory role in early and mature erythroid progenitor proliferation in vitro.

REFERENCES

23. Leonard WJ, Depper JM, Uchiyama T, Smith KA, Waldmann T, Greene WC: A monoclonal antibody that appears to recognize the receptor of human T-cell growth factor; partial characterization of the receptor. Nature 300:267, 1982
The regulatory role of interleukin 2-responsive T lymphocytes on early and mature erythroid progenitor proliferation

Z Estrov, C Roifman, YP Wang, T Grunberger, EW Gelfand and MH Freedman