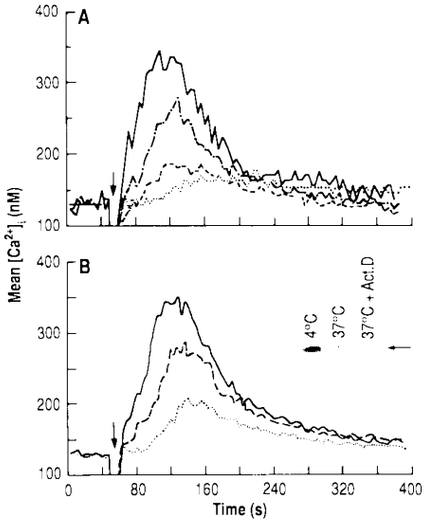

Fig. 4. TCR-induced calcium mobilization in $CD4^+CD8^+$ thymocytes in response to TCR cross-linking by MAb to TCRαβ, and its relation to TCR expression and TCR-ζ phosphorylation. (**A**) Purified B6 $CD4^+CD8^+$ thymocytes were cultured alone for 4 hours in single-cell suspension at either 37°C (solid line) or 4°C (dotted line) and then assessed for TCR-induced calcium mobilization by MAb to TCRαβ (MAb H57-597) (*19*) that was cross-linked by avidin (25 μg) at the indicated times (arrow) (*20*). Alternatively, cells from the same purified B6 $CD4^+CD8^+$ thymocyte populations were cocultured at 37°C with 1×10^6 Ia^+ M12.4.1 (dashed line) or Ia^- M12.C3 (dash-dot) cells, and then assessed for TCR-induced calcium fluxes (*21*). Mean intracellular Ca^{2+} concentrations are displayed versus time. (**B**) Effect of actinomycin D on TCR signaling and TCR-ζ phosphorylation in cultured $CD4^+CD8^+$ thymocytes. Purified B6 $CD4^+CD8^+$ thymocytes were cultured for 4 hours in suspension at either 37°C (solid line) or 4°C (dotted line), or were cultured at 37°C in the presence of actinomycin D (10 μg/ml) (dashed line), and then assessed for TCR-induced calcium mobilization. (Inset) The same cell populations were also assessed for tyrosine-phosphorylated TCR-ζ (arrow) by immunoblotting with antibody to phosphotyrosine (*22*) (Act. D, actinomycin D).

in developing $CD4^+CD8^+$ thymocytes is probably the earliest function performed by CD4 and appears to be unique to immature $CD4^+CD8^+$ thymocytes, as CD4 performs other functions in more differentiated T cells.

REFERENCES AND NOTES

1. P. Kisielow *et al.*, *Nature* **333**, 742 (1988); L. J. Berg *et al.*, *Cell* **58**, 1035 (1989); B. J. Fowlkes, R. H. Schwartz, D. M. Pardoll, *Nature* **334**, 620 (1988); W. C. Sha *et al.*, *ibid.* **336**, 73 (1988); H. R. MacDonald *et al.*, *ibid.*, p. 471.
2. W. L. Havran *et al.*, *Nature* **330**, 170 (1987); T. H. Finkel *et al.*, *ibid.*, p. 179.
3. J. S. Bonifacino *et al.*, *ibid.* **344**, 247 (1990).
4. C. E. Rudd, J. M. Trevillyan, J. D. Dasgupta, L. L. Wong, S. F. Schlossman, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 5190 (1988); A. Veillette, M. A. Bookman, E. M. Horak, L. E. Samelson, J. B. Bolen, *Nature* **338**, 257 (1989); A. Veillette *et al.*, *J. Exp. Med.* **170**, 1671 (1989).
5. T. Nakayama *et al.*, *Nature* **341**, 651 (1989).
6. J. J. Sussman *et al.*, *Cell* **52**, 85 (1988).
7. S. A. McCarthy *et al.*, *Nature* **336**, 76 (1988).
8. P. Marrack *et al.*, *Cell* **53**, 627 (1988).
9. T. Nakayama and A. Singer, unpublished observations.
10. C. Doyle and J. L. Strominger, *Nature* **330**, 256 (1987).
11. I. J. Griffith *et al.*, *J. Exp. Med.* **167**, 541 (1988).
12. T. H. Finkel, P. Marrack, J. W. Kappler, R. T. Kubo, J. C. Cambier, *J. Immunol.* **142**, 3006 (1989); K. Deusch *et al.*, *ibid.* **144**, 2851 (1990).
13. CD3 fluorescence profiles of the CD4 and CD8 thymocyte subpopulations were obtained by computer gating of three-color immunofluorescence data. Staining reagents were fluorescein isothiocyanate (FITC) conjugated to MAb 145-2C11 (anti-CD3) [O. Leo, M. Foo, D. H. Sachs, L. E. Samelson, J. A. Bluestone, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 1374 (1987)]; phycoerythrin (PE) conjugated to MAb GK1.5 (anti-CD4) [D. P. Dialynas *et al.*, *Immunol. Rev.* **74**, 29 (1983)]; biotin conjugated to MAb 53-6.72 (anti-CD8) [J. A. Ledbetter and L. A. Herzenberg, *ibid.* **47**, 63 (1979)]; and Texas red–streptavidin. Leu4-FITC was the control MAb.
14. $CD4^+CD8^+$ thymocytes were isolated before culture by adherence to plates coated with MAb to Lyt2.2, and were >96% $CD4^+CD8^+$ as described (*5*). Nonidet-P40 (NP-40) lysates of cultured thymocytes (2×10^6/lane) were applied to 12.5% SDS–polyacrylamide gel electrophoresis (SDS-PAGE) in nonreducing conditions, subjected to electrotransfer, immunoblotting with MAb to TCRα (MAb H28-710) and to CD3-ε (MAb HMT3-1), and visualization with the use of ^{125}I-labeled protein A [T. Nakayama, R. T. Kubo, H. Kishimoto, Y. Asano, T. Tada, *Int. Immunol.* **1**, 50 (1989); J. S. Spencer and R. T. Kubo *J. Exp. Med.* **169**, 625 (1989)].
15. FU = cell frequency above control × median intensity above control. Median intensity above control was calculated by converting median log channel number above control to linear units with an empirically derived calibration curve for each three-decade logarithmic amplifier used.
16. Harvested cells were stained with both anti-CD3–FITC and 30-H12–biotin (anti-Thy1.2), then Texas red streptavidin. CD3 profiles were obtained by electronic gating on $Thy1.2^+$ cells to exclude the stimulator cells from the FCM analysis.
17. Percent induction is 100 × (change in FU of thymocytes cultured with stimulator cells)/(change in FU of thymocytes cultured alone).
18. The presence of MAb to CD4 or CD8 in these cultures resulted in marked down-modulation of surface CD4 and CD8, respectively, on the harvested cells, which indicated that both antibody preparations were active.
19. R. T. Kubo, W. Born, J. W. Kappler, P. Marrack, M. Pigeon, *J. Immunol.* **142**, 2736 (1989).
20. Cells were incubated with a saturating amount of biotin conjugated to MAb H57-597 (anti-TCRαβ) at 4°C, then warmed to 37°C for 10 min before analysis. Indo-1 Ca^{2+} measurements were done as in P. S. Rabinovitch, C. H. June, A. Grossmann, and J. A. Ledbetter [*J. Immunol.* **137**, 952 (1986)].
21. Stimulator cells were excluded from analysis on the basis of cell size by electronic gating on forward light scatter.
22. Cultured $CD4^+CD8^+$ thymocytes (3×10^7 per lane) were solubilized in 0.5% Triton X-100 lysis buffer with protease and phosphatase inhibitors. After immunoprecipitation with MAb to CD3-ε (MAb 145-2C11), 13% SDS–PAGE in reducing conditions, and electrotransfer, phosphorylated TCR-ζ was detected by immunoblotting with antibodies to phosphotyrosine as described (*5*). Exposure time was 4 days.
23. Both MAb to TCRα (MAb H28-710) and to CD3-ε (MAb HMT3-1) were generated and provided by R. Kubo, Denver, CO. We thank R. Kubo, R. Klausner, and J. Bonifacino for gifts of reagents and helpful discussions.

18 May 1990; accepted 12 July 1990

"Pure" Human Hematopoietic Progenitors: Permissive Action of Basic Fibroblast Growth Factor

MARCO GABBIANELLI,* MASSIMO SARGIACOMO, ELVIRA PELOSI, UGO TESTA, GIANCARLO ISACCHI, CESARE PESCHLE*

Methodology has been developed that enables virtually complete purification and abundant recovery of early hematopoietic progenitors from normal human adult peripheral blood. A fraction of the pure progenitors is multipotent (generates mixed colonies) and exhibits self-renewal capacity (gives rise to blast cell colonies). This methodology provides a fundamental tool for basic and clinical studies on hematopoiesis. Optimal in vitro cloning of virtually pure progenitors requires not only the stimulatory effect of interleukin-3, granulocyte-macrophage colony-stimulating factor, and erythropoietin, but also the permissive action of basic fibroblast growth factor. These findings suggest a regulatory role for this growth factor in early hematopoiesis.

HEMATOPOIESIS IS SUSTAINED BY A pool of stem cells that can self-renew and differentiate into progenitors. These progenitors are committed to specific lineages and are functionally defined as colony- or burst-forming units (CFUs, BFUs), that is, progenitors of the erythroid series (CFU-E, BFU-E), the granulocyte-monocytic lineage (CFU-GM), and multipotent CFU for the GM, erythroid, and megakaryocytic series (CFU-GEMM) (*1*). The progenitors, in turn, differentiate into morphologically recognizable precursors that mature to terminal elements circulating in peripheral blood.

Proliferation and differentiation of early

M. Gabbianelli, M. Sargiacomo, E. Pelosi, U. Testa, C. Peschle, Department of Hematology and Oncology, Istituto Superiore di Sanità, 00161 Rome, Italy.
G. Isacchi, Blood Transfusion Center, University La Sapienza, Rome, Italy.

*To whom correspondence may be addressed.

hematopoietic cells are modulated by specific growth factors (termed hematopoietins), colony-stimulating factors (CSFs), or interleukins (ILs), which are released by accessory cells [monocytes-macrophages, T cells, and large granular lymphocytes (LGLs)] and exert a multi- or uni-lineage stimulus (*2*): thus IL-3 and GM-CSF induce differentiation of pluripotent (CFU-GEMM), early erythroid (BFU-E), and GM progenitors (CFU-GM) (*3*), whereas erythropoietin (Ep), G-CSF, and M-CSF specifically trigger differentiation of late erythroid (CFU-E), granulocytic (CFU-G), and monocytic (CFU-M) progenitors, respectively (*4*).

Three major factors have hindered the analysis of early hematopoiesis. (i) Such analysis requires large amounts of pure hematopoietins, which are now available by recombinant DNA techniques (*2*). (ii) The use of selected fetal calf serum (FCS) batches for hematopoietic culture hindered analysis, because FCS contains unknown hematopoietic stimuli or inhibitors, or both, thus obscuring methodology and interpretation of results (*1*). However, culture conditions have been established without FCS (FCS^-) that allow optimal hematopoietic proliferation and differentiation (*5, 6*). (iii) The coexistence in the cultured hematopoietic population of few progenitors [<1% and <0.1% of bone marrow and peripheral blood cells, respectively (*1*)] amidst a large number of accessory cells releasing unknown quantities of unidentified endogenous growth factors (*1*) masks the effect of exogenous CSFs and hinders both reproducibility and analysis of data. Thus, investigation of early hematopoiesis would be aided by the availability of purified hematopoietic progenitors.

Purification of hematopoietic progenitors is based on methods exploiting the differential densities and membrane antigen expression of progenitors and differentiated lympho-hematopoietic cells (*7*). A combination of these techniques has enabled the stringent purification of hematopoietic stem cells from murine bone marrow (*8, 9*).

We have developed a method to fully purify and abundantly recover hematopoietic progenitors from normal human adult blood. This method is based on three negative and one positive selection steps: step I, isolation of peripheral blood mononuclear cells (PBMCs) on a Ficoll gradient, followed by removal of monocytes by plastic adherence and glutamate dimethylester treatment; step II, isolation of light density cells on a Percoll density gradient; step III, immunoabsorption to magnetic beads coated with a panel of monoclonal antibodies (MAbs) to B, T, and NK cells, monocytes, and granulocytes; and step IV, positive selection by immunoadsorption with two MAbs to CD34 antigen.

The negative selection steps I to III result in the elimination of virtually all differentiated cells from PBMC, including B, T, NK cells, and monocytes [<0.1% for each residual cell population (*10*)]. The frequency of early hematopoietic progenitors (CFU-GEMM + BFU-E + CFU-GM) was evaluated in methylcellulose culture supplemented (FCS^+) or not (FCS^-) with FCS and treated with an optimized mixture of CSFs including IL-3, GM-CSF, and Ep (*11*). In a total of 37 experiments, the mean progenitor frequency in the Ficoll cut (0.06% and 0.07%, respectively) showed an almost 10-fold increase after the Percoll gradient (0.5%) and a 500-fold rise (31% and 33%) after immunodepletion with magnetic beads coated with MAbs (Fig. 1A).

We then selected $CD34^+$ cells within the cell population obtained after the three negative selection steps [25 to 30% of the cells obtained after step III are $CD34^+$ by immunofluorescence flow cytometry (*10*)]. The cells were incubated with two MAbs to CD34 (HPCA-I and BI3C5), washed, and then incubated with magnetic beads coated with antibody to mouse immunoglobulin. Finally, the cells bound to magnetic beads were plated in semisolid cultures for clonogenic assays. After this positive selection, the mean frequency of progenitors (CFU-GEMM + BFU-E + CFU-GM) evaluated in FCS^+ and FCS^- culture increased from 31% and 33% (step III) to 56% and 64% (step IV) (Fig. 1A).

Virtually no progenitors (≤1%) were de-

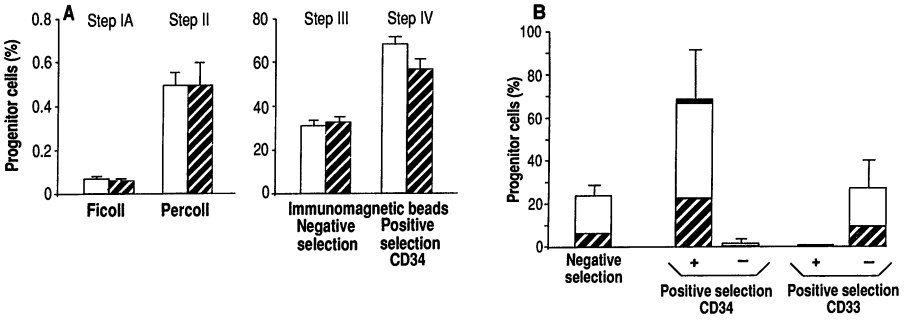

Fig. 1. (**A**) Frequency of hematopoietic progenitors (CFU-GEMM, BFU-E, and CFU-GM) at different steps of purification, assayed in FCS^+ (white bars) or FCS^- (hatched bars) culture (*11*). Mean ± SEM values from a total of 37 separate experiments are presented; the number of separate FCS^+ and FCS^- assays was step I, 30 and 27; step II, 5 and 3; step III, 24 and 22; and step IV, 18 and 9, respectively. **Progenitor purification**. Cells were obtained from the buffy coat of 350 to 400 ml of peripheral blood, collected in preservative-free heparin, from healthy young adult donors after informed consent. **Step IA**. Cells separated over a standard Ficoll-Hypaque density gradient (*d*, 1.077) (Pharmacia Fine Chemicals) were collected, washed twice, and resuspended in Iscove's modified Dulbecco's medium (IMDM). **Step IB**. Peripheral blood mononuclear cells (PBMCs), resuspended in IMDM containing 20% heat-inactivated FCS (Flow Laboratories) (3×10^6 cells/ml) were subjected to two 60-min plastic adherence cycles, washed twice, and then treated with glutamate dimethylester (Sigma) (5×10^{-3} M for 40 min at room temperature) to stringently remove residual monocytes (*6, 21*). The cells were then washed three times and resuspended in IMDM containing 10% FCS (5×10^7 cells/ml). **Step II**. Cells were separated by density centrifugation (600*g* for 30 min at 20°C in 15-ml tubes, 2 to 2.5×10^8 cells per tube) on a three-step discontinuous Percoll gradient (Biochrom KG) (3 ml fractions: *d*, 1.054, 1.066, and 1.077). Lower density cells from fraction 1 (*d*, 1.054), containing the large majority (>70%) of hematopoietic progenitors, were collected, washed three times in IMDM, and resuspended in 3 ml of the same medium. **Step III**. Cells were incubated for 60 min at 4°C with excess amounts (50 μl per 3×10^7 cells) of the following MAbs: OKT3, OKT4, OKT8, OKT11, OKT16, OKM1, OKM5 (Ortho Diagnostic Systems), Leu7, Leu9, Leu11, Leu12, Leu14, Leu19, LeuM1, and LeuM3 (Becton-Dickinson). After three washes with cold IMDM, cells were incubated for 40 min at 4°C with immunomagnetic monodisperse microspheres coated with sheep antibody to mouse immunoglobulin (Ig) G and M (Dynabeads M450, diameter 4.5 μm, 1.3×10^7 particles per milligram, Dynal) at a ratio of five microspheres per cell. The beads, together with rosetting cells, were retained along the tube wall with a magnet for 60 s. Thereafter, the supernatant fluid containing negative cells was recovered. Negative cells were incubated again with immunomagnetic microspheres and processed as above. The cells in the supernatant (negative fraction) were washed twice in cold IMDM, resuspended in 0.5 ml of IMDM, and then processed for either culture in semisolid medium or step IV purification. In steps III and IV, IMDM was supplemented with bovine serum albumin (BSA) (5 mg/ml, >98 to 99% purified, Sigma). **Step IV**. Cells were incubated for 60 min at 4°C with HPCA-I (Becton-Dickinson) and BI3C5 (Sera Lab), as described above, washed, and then incubated with immunomagnetic beads (five microspheres per cell) coated with MAbs to mouse IgG and IgM (Dynal). The beads with rosetting cells were then isolated, counted, and directly plated in semisolid medium for clonogenic assay. In selected experiments, the MAb to CD33 (anti-CD33) My9 (Coulter Clone) was used for positive selection instead of the MAb to CD34 (anti-CD34). (**B**) Purification of human hematopoietic progenitors, as evaluated by their reactivity with anti-CD34 or anti-CD33 MAb. After the three negative selection steps, cells were separated into $CD34^+$ and $CD34^-$ or $CD33^+$ and $CD33^-$ populations according to their reactivity with anti-CD34 or anti-CD33 MAb (see legend to Fig. 1A), and then grown in FCS^- culture (*11*). Mean ± SEM values from three separate experiments are presented.

Table 1. Blast cell colonies generated by step IV cells and secondary colonies after replating (mean ± SEM values from three separate experiments). The assay was performed as in (*13*). Secondary clones comprised all types of colonies (GM, G, M, erythroid bursts, and GEMM clones represented 35%, 10%, 13%, 38%, and 4% of the total number of colonies, respectively). Their size was comparable to that of standard primary culture colonies.

Step IV cells (no./dish)	Primary culture conditions	Blast cell colony no.	Cells/blast cell colony	Secondary colonies/ blast cell colony	Replating efficiency (%)
3×10^3	IL-3 + IL-6	10.3 ± 3.8	27.5 ± 4.3	3.0 ± 0.6	10.8 ± 0.8

tected in the $CD34^-$ cell population (Fig. 1B). We isolated $CD33^+$ and $CD33^-$ cells in three experiments, using the same procedure as for selection of $CD34^+$ cells. All progenitors were observed in the $CD33^-$ fraction; virtually none were detected among $CD33^+$ cells (Fig. 1B).

In conclusion, the isolated peripheral blood progenitors are $CD34^+CD33^-$, in contrast to the $CD34^+CD33^+$ majority of bone marrow progenitors (*12*). The $CD34^+CD33^-$ population is apparently more immature, for the following reasons. (i) They generate CFU in long-term marrow culture, whereas $CD34^+CD33^+$ progenitors do not (*12*). (ii) A fraction of $CD34^+CD33^-$ progenitors purified from peripheral blood is multipotent [gives rise to mixed colonies (Fig. 1B)] and exhibits self-replicating activity [generates blast cell colonies (*13*), that in turn give rise to secondary clones of G, M, GM, erythroid, and GEMM type when replated in semisolid culture (Table 1)]. These findings indicate that the purified circulating $CD34^+CD33^-$ cells are early hematopoietic progenitors, a fraction of which are multipotent and exhibit self-renewal capacity, that is, are endowed with stem cell properties. In support of these observations, circulating autologous progenitors can reconstitute the human lympho- and hematopoietic systems in patients treated with high-dose antiblastic agents for a variety of malignant diseases (*14*).

The final progenitor frequency rarely exceeded 70%. This purification threshold may be hypothetically attributed to three alternative or complementary mechanisms. (i) A minority of the purified $CD34^+$-$CD33^-$ cells are not hematopoietic progenitors. (ii) Some $CD34^+CD33^-$ progenitors are functionally damaged during the purification procedure and become unresponsive to CSFs (*7, 8*). (iii) A minority of purified $CD34^+CD33^-$ progenitors are physiologically unresponsive to conventional hematopoietic CSFs and require the stimulus of other growth factors.

To test the third hypothesis, we screened a variety of growth factors [acidic or basic fibroblast growth factor (aFGF, bFGF), platelet-derived growth factor, epidermal growth factor, insulin-like GF-1, and transforming growth factor–β2 (TGF-β2) (*15*)] for their possible stimulatory effect on step IV progenitors treated with IL-3, GM-CSF, and Ep, as evaluated by clonogenetic capacity in semisolid culture (*15*).

Only bFGF exerted a significant stimulatory effect. In clonogenetic culture of step IV progenitors, graded amounts of bFGF induced a stepwise potentiating effect on the action of IL-3 + GM-CSF + Ep, as evaluated by the number of erythroid bursts and GM colonies per dish, and the number of cells per burst and GM colonies (Fig. 2A). A plateau dosage (100 ng/ml) of bFGF alone was ineffective, but combined treatment with IL-3 + GM-CSF + Ep resulted in optimal growth of the progenitors: their mean frequency rose to 92.3% (range, 79 to 98%) and 87.5% (range, 80.5 to 97%) in FCS^+ and FCS^- cultures, respectively (Fig. 2B).

These experiments indicate that bFGF, although by itself ineffective, exerts a permissive effect by inducing a significant number of early hemopoietic progenitors to respond to multilineage hemopoietins (IL-3 + GM-CSF) in clonogenic culture.

The purification index in the step IV fraction does not reach the theoretical value of 100%. However, the monitored frequency (92.3%) is seemingly underestimated: (i) the plateau bFGF dosage (100 ng) does not exert the peak stimulatory effect in all experiments (see legend to Fig. 2A). (ii) A minority (5 to 10%) of progenitors may be either functionally damaged during the purification procedure or unresponsive to the growth factors we used [IL-6 and G-CSF may have an additive effect (see legend to Fig. 2B)].

The recovery of progenitors (CFU-GEMM + BFU-E + CFU-GM) after the purification steps, expressed as percentage of those present in PBMCs (step I), was 76.2 ± 4.9% and 71.3 ± 4.7% for step II and step III fractions, respectively, and 20.2 ± 2.9% for step IV cells (*16*).

This methodology provides a fundamental tool to elucidate the cellular and molecular basis of early hematopoiesis. The availability of a large number of purified peripheral blood progenitors may also be useful for clinical transplantation. Peripheral blood stem cells are more desirable for autologous transplantation, because they are more

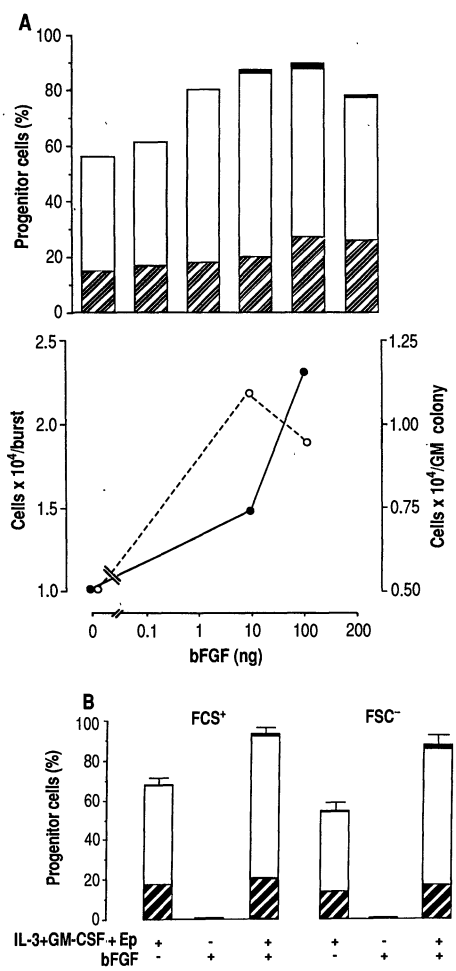

Fig. 2. (**A**) Effect of combined addition of graded doses of recombinant bovine bFGF (specific activity, 5×10^7 U/ng, Amgen) and plateau dosages of IL-3 + GM-CSF + Ep on the cloning of step IV cells in FCS^+ culture (see also Fig. 1A), as evaluated by the number of GEMM, BFU-E, and GM colonies per dish (top panel, black, white, and hatched bar portions, respectively) and cells per erythroid bursts and GM colonies (bottom). A representative experiment is shown here; in some experiments, the peak effect of bFGF was observed at a dosage lower than 100 ng/ml. (**B**) Effect of a plateau dosage of bFGF (100 ng/ml) on the colony formation efficiency of step IV $CD34^+$ progenitors (CFU-GEMM, BFU-E, and CFU-GM, black, white, and hatched bar portions, respectively) in FCS^+ or FCS^- culture, supplemented or not with peak dosages of IL-3 + GM-CSF + Ep (see also Fig. 1A). Mean ± SEM values are presented from 18 and 9 $bFGF^-$ experiments (Fig. 1A), as compared to 6 and 4 $bFGF^+$ experiments in FCS^+ and FCS^- cultures, respectively. In two FCS^+ culture experiments, further addition of IL-6 (10 ng/ml) to $bFGF^+$ dishes raised the frequency of progenitors from 95% and 96% to 99% and 98%, respectively. bFGF (100 ng/ml) also induced a significant rise of the frequency of progenitors (CFU-GEMM + BFU-E + CFU-GM) in the step III fraction assayed in FCS^- culture, that is, from 32.5 ± 3.0 in $bFGF^-$ dishes (see Fig. 1A) to 44.1 ± 5.5 in $bFGF^+$ dishes from four separate experiments.

readily available and can more rapidly reconstitute lympho-hematopoiesis than bone marrow stem cells (*17*).

These observations suggest a role for bFGF in hematopoiesis. This growth factor, produced by a variety of cell types (fibroblasts, endothelial cells, and macrophages), is mitogenic to many cells, including fibroblasts and capillary endothelial cells (*18*). In *Xenopus* blastula it can induce cells from the animal pole to develop as mesoderm (*19*) and as ventral derivatives, including blood cells. Our studies indicate a synergistic interaction between bFGF and multilineage CSFs (IL-3, GM-CSF) on early adult hematopoietic progenitors; both CSFs and bFGF have been postulated to act on target cells after binding to the extracellular matrix, particularly heparan sulfate (*20*). We suggest that bFGF may have a role in the control of proliferation or differentiation, or both, of early adult hematopoietic progenitors, as it apparently does in embryonic hematopoietic development in *Xenopus* (*19*).

REFERENCES AND NOTES

1. D. Metcalf and M. A. S. Moore, *Haemopoietic Cells* (North-Holland, Amsterdam, 1971); C. Peschle *et al.*, in *Current Concepts in Erythropoiesis*, C. D. R. Dunn, Ed. (Wiley, London, 1983), pp. 339–387; D. Metcalf, *The Hemopoietic Colony Stimulating Factors* (Elsevier, Amsterdam, 1984); L. Sachs, *Science* **238**, 1374 (1987); D. Metcalf, *Nature* **339**, 27 (1989).
2. Y. C. Yang *et al.*, *Cell* **47**, 3 (1986); G. G. Wong *et al.*, *Science* **228**, 810 (1985); K. Jacobson, *Nature* **313**, 806 (1985); D. Metcalf, *Science* **229**, 16 (1985); S. C. Clark and R. Kamen, *ibid.* **236**, 1229 (1987).
3. C. E. Müller-Sieburg *et al.*, *J. Exp. Med.* **167**, 1825 (1988); Y. Sonada *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 4360 (1988); A. G. Leary *et al.*, *Blood* **70**, 1343 (1987); R. E. Donahue *et al.*, *ibid.* **66**, 1479 (1985).
4. C. J. Evans and A. C. Eaves, *Blood* **52**, 1196 (1978); D. Caracciolo *et al.*, *J. Exp. Med.* **166**, 1851 (1987).
5. J. F. Eliason, *J. Cell. Physiol.* **128**, 231 (1986); D. L. McLeod, M. M. Shreeve, A. A. Axelrad, in *In Vitro Aspects of Erythropoiesis*, M. J. Murphy, Ed. (Springer-Verlag, New York, 1987), pp. 31–36.
6. M. Valtieri *et al.*, *Blood* **74**, 460 (1989); M. Gabbianelli *et al.*, *ibid.*, p. 2657.
7. D. C. Linch and D. G. Nathan, *Nature* **312**, 775 (1984); J. Horiguchi, M. K. Warren, D. Kufe, *Blood* **69**, 1259 (1987); L. Iu *et al.*, *J. Immunol.* **139**, 1823 (1987); W. Oster *et al.*, *Blood* **70**, 1700 (1987); T. Otsuka *et al.*, *J. Immunol.* **140**, 2288 (1988); C. A. Sieff *et al.*, *Blood* **72**, 1316 (1988); E. Vellenga *et al.*, *ibid.* **71**, 1529 (1988).
8. G. J. Spangrude, S. Heimfeld, I. L. Weissman, *Science* **241**, 58 (1988).
9. G. J. Spangrude, C. E. Müller-Sieburg, S. Heimfeld, I. L. Weissman, *J. Exp. Med.* **167**, 1671 (1988).
10. We evaluated expression of cell surface markers by indirect immunofluorescent flow cytometry. Cells (1×10^5) were incubated with MAbs Leu4, Leu12, Leu19, LeuM3, T9 (anti-transferrin receptor) BI3C5, HPCA-I, or antibody to HLA-DR (Becton-Dickinson). After three washes and incubation with fluorescein isothiocyanate-conjugated $F(ab')_2$ fragments of goat antibody to mouse immunoglobulin, cells were washed, resuspended in Hank's formalin solution, and analyzed by FACScan (Becton-Dickinson).
11. The clonogenic assays were performed as follows. Step I progenitors. In FCS^+ cultures, PBMCs (step IA) were cultured (3×10^5 cells/ml per dish at least two plates per point) in 0.9% methylcellulose, 40% FCS (Flow), and Ep (3.0 IU/ml) in Iscove's modified Dulbecco's medium supplemented with γ-thioglycerol (10^{-4} M) (Sigma) at 37°C in a humidified atmosphere of 5% CO_2/5% O_2/90% air. Colony scoring was performed on day 14 of culture (*6*). For FCS^- cultures, FCS was replaced by a mixture of (i) >98 to 99% purified BSA, (ii) human low-density lipoprotein, (iii) pure human transferrin, and (iv) a mixture of pancreatic bovine insulin, nucleosides, rare inorganic elements, sodium pyruvate, and L-glutamine (*6*). The dishes (at least two plates per point) were incubated as for FCS^+ cultures. Colonies were scored 1 to 2 days later than FCS^+ culture (*6*). In step II to step IV progenitors, the FCS^+ and FCS^- culture conditions were as described above, with the following exceptions. (i) Progenitors were seeded at a concentration of 1×10^5 (step II) or 1×10^2 (step III and step IV) cells/ml per dish. In step III and step IV assays, control unicellular cultures provided equivalent results. (ii) Several CSFs were added at optimal concentrations to sustain the formation of CFU-GEMM, BFU-E, and CFU-GM colonies, that is, IL-3 (100 U/ml), GM-CSF (10 ng/ml), and Ep (3.0 U/ml). Control experiments were performed with addition of IL-3 and GM-CSF only. In experiments presented in Fig. 2, A and B, bFGF was also added at the indicated concentration.
12. L. C. Strauss *et al.*, *Exp. Hematol. (N.Y.)* **14**, 878 (1986); F. E. Katz, R. Tindle, R. P. Sutherland, M. F. Greaves, *Leuk. Res.* **9**, 191 (1985); R. G. Andrews, J. W. Singer, I. D. Bernstein, *Blood* **67**, 842 (1986); *J. Exp. Med.* **169**, 1721 (1989).
13. A. G. Leary, Y. Hirai, T. Kishimoto, S. C. Clark, M. Ogawa, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 4535 *1*(1989).
14. D. M. Carthy and J. M. Goldman, *CRC Crit. Rev. Clin. Lab. Sci.* **20**, 1 (1984); A. J. Bell *et al.*, *Bone Marrow Transplant.* **1**, 103 (1986); L. B. To and C. A. Juttner, *Br. J. Haematol.* **66**, 285 (1987); C. A. Juttner *et al.*, *Transplant. Proc.* **20**, 40 (1988); A. Kessinger *et al.*, *Blood* **71**, 723 (1988).
15. The dose-response curves were performed in FCS^- clonogenic culture seeded with step IV cells treated with recombinant aFGF (R&D Systems) (0.1 to 200 ng/ml), recombinant platelet-derived growth factor (Amgen) (0.1 to 10 ng), recombinant epidermal growth factor (Peninsula Laboratories) (1 to 100 ng), recombinant insulin-like GF-1 (Amgen) (1 to 20 ng), or transforming GF-β2 (>95% purified, R&D Systems) (0.01 to 10 ng).
16. In a typical experiment on the buffy coat from a single donor, the cell number and progenitor yield were 8×10^8 cells and 100% yield (step IA), 0.48×10^8 and 79% (step II), 8×10^5 and 77% (step III), and 1.0×10^5 and 33% (step IV).
17. O. Prummer *et al.*, *Exper. Hematol.* **13**, 891 (1985); L. B. To *et al.*, *Bone Marrow Transplant.* **2**, 111 (1987); A. Kessinger *et al.*, *Blood* **74**, 1260 (1989).
18. L. Schweigerer *et al.*, *Nature* **325**, 257 (1987); J. J. Feige and A. Baird, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 3174 (1989); D. B. Rifkin and D. Moscatelli, *J. Cell Biol.* **109**, 1 (1989).
19. J. M. Slack *et al.*, *Nature* **326**, 197 (1987); J. M. Slack *et al.*, *Development* **105**, 147 (1989); D. Kimel and M. Kirschner, *Cell* **51**, 869 (1987); D. Kimelmann *et al.*, *Science* **242**, 1053 (1988).
20. M. Y. Gordon *et al.*, *Nature* **326**, 403 (1987); W. H. Burgess and T. Maciag, *Annu. Rev. Biochem.* **58**, 575 (1989); R. Roberts *et al.*, *Nature* **332**, 376 (1988); I. J. Vlodasky *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 2292 (1987).
21. D. L. Thiele and P. R. Lipsky, *J. Immunol.* **134**, 786 (1985).

22. We thank S. Peddis for technical assistance, F. Cionni and M. E. Ganci for secretarial assistance, and D. Marinelli for editorial help and graphics. Supported in part by Progetto Italia-USA Terapia dei Tumori, Istituto Superiore di Sanità, Rome, and Progetto finalizzato Biotechnologie (grant no. 89.00256.70 to C.P.), CNR, Rome, Italy. Recombinant human IL-3 (specific activity, 4×10^6 U/mg) and GM-CSF (1.7×10^7 U/mg) were supplied by S. Clark (Genetics Institute, Pilot Development Laboratory). Recombinant human Ep (1.1×10^5 U/mg) was provided by Amgen.

12 February 1990; accepted 5 July 1990

Protection from Chemotherapy-Induced Alopecia in a Rat Model

ATIF M. HUSSEIN, JOAQUIN J. JIMENEZ, CATHERINE A. MCCALL, ADEL A. YUNIS*

Alopecia (hair loss) is among the most distressing side effects of cancer chemotherapy. Little progress has been made, however, in its prevention or treatment, partly because of the lack of suitable experimental model. In recent work on the treatment of myelogenous leukemia in the rat, the following observations were made: (i) treatment of 8-day-old rats with cytosine arabinoside consistently produced alopecia, and (ii) ImuVert, a biologic response modifier derived from the bacterium *Serratia marcescens*, uniformly produced complete protection against the alopecia. In subsequent experiments, both cyclophosphamide and doxorubicin also produced alopecia in this model, and the doxorubicin-induced alopecia was prevented by treatment with ImuVert. The potential relevance of these observations to chemotherapy-induced alopecia in the clinical setting should be examined.

ALOPECIA IS A FREQUENT AND DISTRESSING side effect of many clinically active chemotherapeutic agents, often causing patients to refuse potentially curative chemotherapy (*1–5*). Although this complication has been known for many decades (*3*), little progress has been made in its prevention or treatment (*6–9*), in part because of a lack of a suitable, reproducible, experimental model. Although doxorubicin-induced hair loss has been described in the Angora rabbit (*10*), its severity is assessed by weighing the amount of hair that regrows after shaving or the amount of hair shed upon grooming. Recently we have been studying the efficacy of chemotherapy in