References and Notes

1. H. Sonnenburg *et al.*, *Can. J. Physiol. Pharmacol.* **60**, 1149 (1982); M. G. Currie *et al.*, *Science* **221**, 71 (1983); R. T. Grammer *et al.*, *Biochem. Biophys. Res. Commun.* **116**, 696 (1983); G. Thibault *et al.*, *Hypertension* **5** (Suppl. 1), 81 (1983).
2. M. G. Currie *et al.*, *Science* **223**, 67 (1984).
3. R. E. Lang *et al.*, *Nature (London)* **314**, 264 (1985).
4. C. E. Seidman *et al.*, *Science* **225**, 324 (1984).
5. M. Yamanaka *et al.*, *Nature (London)* **309**, 719 (1984).
6. M. Maki *et al.*, *ibid.*, p. 722.
7. K. Nakayama *et al.*, *ibid.* **318**, 699 (1984).
8. J. F. Habener *et al.*, in *Peptides: Synthesis-Structure-Function, Proceedings of the Seventh American Peptide Symposium*, D. H. Rich and E. Gross, Eds. (Pierce Chemical, Rockford, Ill., 1981), p. 457.
9. K. Kangawa *et al.*, *Nature (London)* **312**, 152 (1984).
10. T. G. Flynn *et al.*, *Science* **228**, 323 (1985).
11. N. C. Trippodo *et al.*, *Biochem. Biophys. Res. Commun.* **119**, 282 (1984).
12. I. Tanaka, K. S. Misono, T. Inagami, *ibid.* **124**, 663 (1984).
13. I. Harary and B. Farley, *Exp. Cell Res.* **29**, 451 (1963).
14. M. Cantin *et al.*, *Science* **214**, 569 (1981).
15. M. Cantin *et al.*, *Histochemistry* **80**, 113 (1984); J. Metz, V. Mutt, W. G. Forssman, *Anat. Embryol. (Berlin)* **170**, 123 (1984).
16. J. A. Scott *et al.*, *Circ. Res.* **56**, 72 (1985).
17. Preparing cardiocyte cultures: 1-day-old rats were killed with ether anesthesia. Hearts were excised and transversely sectioned; sections containing atria were dispersed in 0.85 mg of trypsin per milliliter with 0.2 mg/ml EDTA, plated in Dulbecco's minimal essential medium without glutamine (Gibco), supplemented with 15 percent Nu-Serum (Collaborative Research) and 0.6 mg/ml thymidine, and placed at 37°C in a 5 percent CO_2 atmosphere (*16*). Ten neonatal rats yielded 0.5×10^7 to 1×10^7 cells. Radiolabeling cardiocytes: cultured cardiocytes were incubated in 1 ml of RPMI 1640 (Gibco) without either cysteine or Nu-Serum for 30 minutes. The culture medium was replaced, and 100 μCi of [^{35}S]cysteine (1000 Ci/mmol) was added. After a 3-hour incubation, the medium was harvested and centrifuged to remove cellular material. Nonidet P-40, Tris *p*H 7.4, aprotinin, and phenylmethylsulfonyl fluoride (PMSF) were added to the culture medium to yield final concentrations of 0.5 percent, 10 m*M*, 100 Kallikrein inactivated units (KIU) per milliliter, and 0.5 m*M*, respectively. Immunoprecipitation of proANF: immunoprecipitations were performed in phosphate-buffered saline with 0.1 percent Nonidet P-40, 100 μg of bovine serum albumin per milliliter, 100 KIU/ml aprotinin, and PMSF 0.5 m*M*. Atriopeptin III (Fig. 1), iodinated by the chloramine-T method [W. M. Hunter and F. C. Greenwood, *Nature (London)* **194**, 495 (1962)], or culture medium from [^{35}S]cysteine-labeled cardiocytes were incubated with antiserum for 1 hour at 21°C and 3 hours at 4°C. Antigen-antibody complexes were precipitated by incubation with goat antiserum to rabbit gammaglobulin at 4°C for 2 hours [S. G. Rockson, C. J. Homcy, E. Haber, *Circ. Res.* **46**, 808 (1980)]. The precipitates were solubilized and boiled in 3 percent sodium dodecyl sulfate (SDS) and 5 percent β-mercaptoethanol. Proteins were electrophoresed on a 17 percent polyacrylamide gel containing SDS; gels were dried and analyzed by autoradiography. The molecular sizes of the radiolabeled peptides fractionated on these gels were estimated by comparison with the electrophoretic mobility of protein standards (Bethesda Research Laboratories).
18. L. A. Sternberger, *Immunohistochemistry* (Wiley, New York, ed. 2, 1979).
19. K. Y. Hui, E. Haber, G. R. Matsueda, *Science* **222**, 1129 (1983).
20. B. A. Khaw *et al.*, *Science* **217**, 1050 (1982).

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Human Recombinant Granulocyte-Macrophage Colony-Stimulating Factor: A Multilineage Hematopoietin

Abstract. *Human recombinant granulocyte-macrophage colony–stimulating factor (GM-CSF) was tested for its ability to induce colony formation in human bone marrow that had been enriched for progenitor cells. In addition to its expected granulocyte-monocyte colony-stimulating activity, the recombinant GM-CSF had burst-promoting activity for erythroid burst-forming units and also stimulated colonies derived from multipotent (mixed) progenitors. In contrast, recombinant erythroid-potentiating activity did not stimulate erythroid progenitors. The experiments prove that human GM-CSF has multilineage colony-stimulating activity.*

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The survival, proliferation, and terminal differentiation of hematopoietic progenitors in vitro depends on cellular hematopoietic growth factors, also known as colony-stimulating factors (CSF's) (*1*). These factors are usually classified by the types of mature cells found in the colonies to which they give rise in response to the differentiation process they stimulate. Two murine factors have been purified to homogeneity, and their genes have been cloned and sequenced. One, interleukin-3 (IL-3) (*2*), influences multipotent (that is, mixed) progenitors and those with restricted lineage. This single factor has burst-promoting activity (BPA) for immature erythroid burst-forming units (BFU-E) and also stimulates the formation of granulocyte-macrophage colonies. A second murine factor, granulocyte-macrophage colony-stimulating factor (GM-CSF) (*3*), is required for the growth of granulocyte-macrophage colonies (CFU-GM). Both of these nonhomologous murine factors are now thought to be derived from T lymphocytes, but the full range of cells capable of their expression has not been determined.

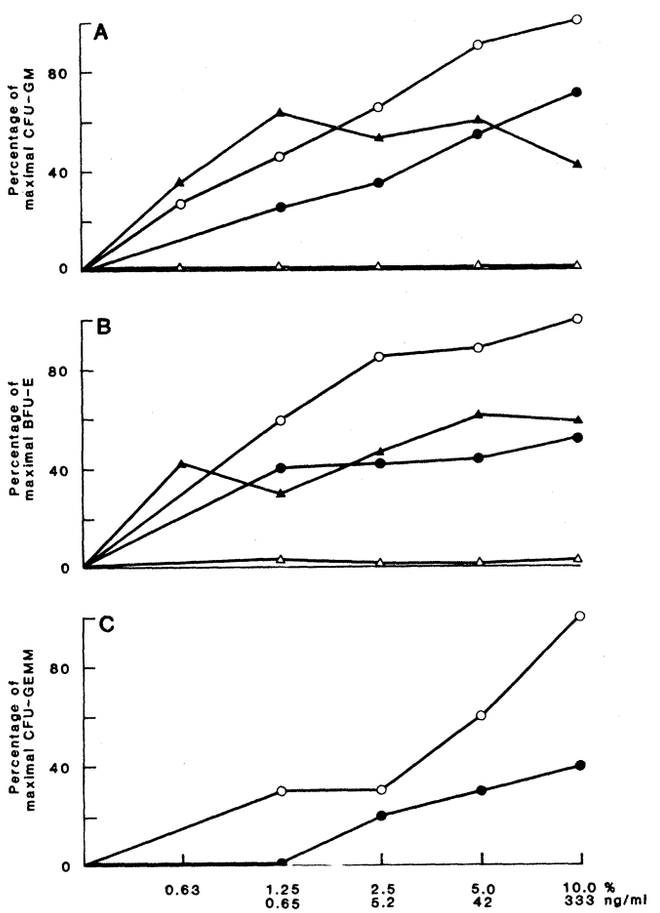
Little is known about the biochemistry and biological activities of highly purified human CSF's. Both BPA and GM-CSF are produced by various human cell types, including T-lymphocytes (*4*) and monocytes (*5*). Until recently, neither factor had been purified to homogeneity, but studies of partially purified factors have not convincingly demonstrated that they have separate activities (*6*). The human GM-CSF gene was recently cloned from a Mo cell expression library (*7*). This human gene shows approximately 60 percent homology with its murine counterpart. We now report that recombinant human GM-CSF not only stimulates granulocyte-macrophage formation, but also has burst-promoting activity and stimulates the formation of multipotent colonies containing granulocytes, erythroid cells, monocytes, and occasionally, megakaryocytes.

To obtain complementary DNA (cDNA) clones that express biologically active human GM-CSF, we prepared cDNA's from membrane-bound messenger RNA's of lectin-stimulated Mo cells, constructed cDNA libraries in expression vectors, and screened the resulting plasmid pools by transient expression in monkey COS-1 cells (*7*). One of the plasmid vectors positive for GM-CSF in the screening assay was introduced by DNA transfection into a large number of COS-1 cells, which were then allowed to condition medium in the absence of serum. The recombinant GM-CSF produced in this manner had physical properties virtually identical to those of the natural protein obtained from Mo cell–conditioned medium (Mo-CM) (*7*) and was purified by gel filtration followed by reverse-phase high-pressure liquid chromatography (HPLC). The purified protein migrated as a single heterogeneous band with a molecular mass of 18 to 24 kD when analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and was estimated to be over 95 percent pure. The specific activity of the purified protein was 1×10^7 to 4×10^7 units per milligram in an agar bone marrow CFU-GM assay. (One unit is defined as the amount of GM-CSF that stimulates the formation of one colony per 10^4 cells above the background level when CSF is below saturation level.)

Table 1. Monoclonal antibodies used to enrich progenitors from bone marrow (*8, 19*).

Antibody	Specificity
Leu 1	T lymphocyte
Leu 5b	T lymphocyte, some natural killer (NK) cells
Campath 1	B and T lymphocytes
TG1	Myeloid maturation
Leu M1	Monocytes, granulocytes
Mo 1	Monocytes, myeloid maturation, NK cells
My 8	Monocytes, myeloid maturation
YTH 89.18	Erythroid
LICR.LON.R18	(Glycophorin A)

Human bone marrow cells were obtained from adult volunteers, and the hematopoietic progenitors among the bone marrow cells were enriched by immune panning with monoclonal antibodies directed against myeloid, erythroid, and lymphoid maturation antigens (*8*) (see Table 1). The hematopoietic progenitors were then cultured in semisolid medium (*9*). As expected (Fig. 1A), the formation of granulocyte-macrophage colonies showed a characteristic dose-dependent response to Mo-CM and, as previously demonstrated, the recombinant GM-CSF present in either COS-1–conditioned medium (COS-CM) or purified by high-performance liquid chromatography (HPLC-GM-CSF) also stimulated granulocyte-monocyte colony formation in a dose-dependent fashion. COS-CM (but not HPLC-GM-CSF) showed a plateau in biological activity at 60 percent of the maximum observed with Mo-CM. Mo-CM, however, did not plateau at the maximum concentration possible (10 percent) in this assay. Figure 1B shows the dose dependency of BFU-E observed when erythropoietin was added to the culture plates on day 3. In this assay, virtually no background BFU-E–derived colonies formed in the absence of BPA, and BFU-E showed complete dependence on added BPA for erythroid colony formation in culture. GM-CSF from COS-1 cell–conditioned medium and GM-CSF from the HPLC fraction were equally effective and saturating as sources of BPA, but again were not as active as Mo-CM. BFU-E were of a similar size range in all three assays. The failure of HPLC-GM-CSF to saturate CFU-GM, in contrast to BFU-E, suggests that of CFU-GM require higher concentrations of GM-CSF than BFU-E, but this could be a result of suboptimal CFU-GM assay conditions in methylcel-



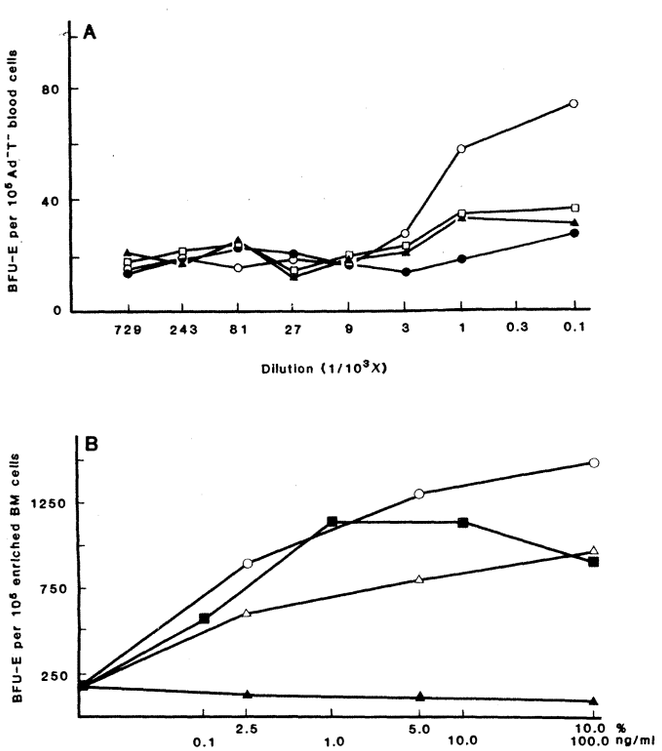


Fig. 1 (left). Normalized response of (A)CFU-GM and (B)BFU-E to increasing doses of Mo-CM (○), COS-CM (▲), HPLC-GM-CSF (●), and mock COS (△) from two experiments. Maximum colony number stimulated by 10 percent Mo-CM was 1427 CFU-GM and 1970 BFU-E per 10^5 nonadherent antibody-negative (Ad^- Ab^-) bone marrow (BM) cells, respectively. (C) Mixed-colony (CFU-GEMM) response to increasing doses of Mo-CM (○) and HPLC-GM-CSF (●) (one experiment). Maximum colony number stimulated by 10 percent Mo-CM was 100 multipotent progenitors per 10^5 Ad^- Ab^- BM cells. The concentrations of growth factor tested ranged from 0.63 to 10 percent of the final volume for Mo-CM and COS-CM and from 0.65 to 333 ng/ml for the HPLC-GM-CSF. The values of Mo-CM were higher than those for COS-CM for all dosage levels by the Wilcoxon nonparametric signed-rank sum test when two experiments for each progenitor class were run in STAT80 (Salt Lake City) (BFU-E and CFU-GM, $P < 0.02$; multipotent progenitors, $P < 0.05$). Fig. 2 (right). (A) Results of an experiment showing the response of blood BFU-E to Mo-CM and erythroid potentiating activity. Mo-CM (○) shows significant BPA activity, whereas no activity above background is seen with purified EPA (●), recombinant COS-EPA (▲), or diluent (phosphate-buffered saline containing 1 percent bovine serum albumin) (□). Ad^-T^- cells are nonadherent and T-lymphocyte-depleted. (B) Results of an experiment showing the response of enriched bone marrow BFU-E to Mo-CM, GM-CSF, and EPA. Mo-CM (○) shows significant activity. Approximately 50 percent of this activity is seen with HPLC-GM-CSF (■) and COS-GM-CSF (△). COS-EPA (▲) shows no activity.

lulose. As was true for BFU-E, mixed colonies of granulocytes, erythroblasts, macrophages, and occasional megakaryocytes showed similar dose responses to Mo-CM and HPLC-GM-CSF (Fig. 1C).

Westbrook *et al.*, (*10*) reported that the erythroid-potentiating activity (EPA) present in Mo-CM, recently purified to homogeneity and cloned from a Mo-cell cDNA library (*11*), enhances both BFU-E– and CFU-E–derived colony formation above background in a semisolid culture system with a reduced amount of fetal calf serum. When nonadherent blood mononuclear cells depleted of T lymphocytes were used as target cells (*12*) in the full-serum BPA assay, no activity of purified or recombinant EPA was detectable over a wide concentration range, in contrast to the readily detectable BPA present in crude Mo-CM (Fig. 2A). Similarly, no response was observed when EPA was tested on enriched bone marrow target cells (Fig. 2B) in an assay identical to the one used to test the recombinant GM-CSF. These experiments demonstrate that the Mo cell line expresses at least two different erythropoietic regulatory genes. One of these, GM-CSF, is active in a full-serum assay system. The other, EPA, is not active in a full-serum assay system that readily detects BPA.

That purified recombinant GM-CSF has trifunctional activity explains the difficulty of separating its activities biochemically, but also raises other questions. The DNA sequence of human GM-CSF is approximately 60 percent homologous with the DNA sequence of murine GM-CSF, but has no homology with the murine IL-3 sequence (7). Human GM-CSF may therefore be a functional but not a structural equivalent of murine IL-3. Welte *et al.* (*13*) recently purified to apparent homogeneity a pluripotent CSF from the human bladder carcinoma cell line 5637 with physical properties similar to those of GM-CSF. A comparison of amino acid sequence of this protein with that of GM-CSF will determine whether it is the same or a different protein. Whether GM-CSF is important for the survival and differentiation of pluripotent stem cells and progenitor cells of other hematopoietic lineages is not presently known, but multipotent colonies were observed in cultures containing GM-CSF.

Although murine GM-CSF was originally thought to stimulate granulocyte-macrophage progenitors only, the purified hormone was shown to initiate the proliferation of multipotent and erythroid progenitors present in murine fetal liver cultures (*3, 14*). In those experiments, terminal differentiation to hemoglobin-containing colonies required spleen-conditioned medium. Whether this is due to erythropoietin or some other factor in the spleen-conditioned medium or serum used to support the cultures has not been resolved. Our experiments show that human recombinant GM-CSF and erythropoietin alone can stimulate the entire differentiation program of human BFU-E and of multipotent progenitors in serum. The effect is not due to a contaminating activity in erythropoietin. Indeed, we have recently made similar observations using both recombinant GM-CSF and recombinant erythropoietin (*15*). We also observed that recombinant GM-CSF stimulated approximately half as many colonies, derived from BFU-E, CFU-GM, and multipotent progenitors, as did Mo-CM. A factor (or factors) present in Mo-CM therefore appears to induce the development of a subset of progenitor-derived colonies that are unaffected by GM-CSF.

We did not determine whether GM-CSF acts directly or indirectly on erythroid and multipotent progenitor cells. Our data suggest that its action is direct, since highly enriched progenitors, depleted of T lymphocytes and monocytes, were cultured at very low densities in these experiments. We have now obtained similar results with highly enriched fetal liver progenitors (*16*), but whether fetal and adult cells respond to GM-CSF by the same mechanism requires further investigation. It is possible that GM-CSF acts indirectly through other accessory cells. This question may be resolved by the use of limiting dilution techniques. (*17*).

Finally, these experiments may bear on current clinical studies of the role of T-cell depletion in the prevention of graft-versus-host-disease (*18*). GM-CSF is a T-cell product with multiple CSF activity. It may be required in the early stages of recovery after marrow transplantation. Exhaustive depletion of T lymphocytes could therefore lead to failure of engraftment. Indeed, recent clinical experience suggests that this may occur (*18*).

References and Notes

1. A. W. Burgess and D. Metcalf, *Blood*, **56**, 947 (1980); N. A. Nicola and M. Vadas, *Immunol. Today* **5**, 76 (1984).
2. N. J. Ihle, J. Keller, L. Henderson, *J. Immunol.* **129**, 2431 (1982); M. C. Fung *et al.*, *Nature (London)* **307**, 233 (1984); D. M. Rennick *et al.*, *J. Immunol.* **134**, 910 (1985); N. J. Ihle, *ibid.* **131**, 282 (1983).
3. A. W. Burgess, J. Camakaris, D. Metcalf, *J. Biol. Chem.* **252**, 1998 (1977); N. M. Gough *et al.*, *Nature (London)* **309**, 763 (1984).
4. D. G. Nathan *et al.*, *J. Exp. Med.* **147**, 324 (1978); B. Torok-Storb *et al.*, *Blood* **58**, 171 (1981).
5. L. I. Gordon *et al.*, *Blood* **55**, 1047 (1980); C. P. L. Reid *et al.*, *Br. J. Haematol.* **48**, 155 (1981); K. S. Zuckerman, *J. Clin. Invest.* **67**, 702 (1981).
6. N. A. Nicola *et al.*, *Blood* **54**, 614 (1979); T. Hoan *et al.*, *ibid.* **61**, 960 (1983).
7. G. G. Wong *et al.*, *Science* **228**, 810 (1985).
8. Normal bone marrow cells from adult volunteers were separated on Ficoll-Paque (1.077 g/cm^3) (Pharmacia), and monocytes were depleted by overnight adherence to 60-mm tissue culture dishes (Lux, Miles Laboratories) at 37°C. The nonadherent cells were incubated for 30 minutes at 4°C with optimal concentrations of the antibodies, washed, and incubated for 1 hour at 4°C on petri dishes coated with rabbit antibody to mouse immunoglobulin. The nonadherent antibody-negative cells were removed by swirling the plates gently and washing twice with medium. Of the starting bone marrow cells, 3 to 5 percent were recovered with a plating efficiency of 2.2 to 2.4 percent for BFU-E, and 1.2 to 1.7 percent for CFU-GM. BFU-E and CFU-GM were recovered at 58 to 64 percent and 22 to 55 percent, respectively, and the final fraction contained fewer than 1 percent lymphocytes and 0 to 5 percent monocytes. Most of the cells in this population were myeloblasts and large mononuclear cells with prominent nucleoli, indented or oval nuclei, and basophilic fairly abundant cytoplasm. Variable numbers of promyelocytes and myelocytes contaminated this fraction.
9. Purified bone marrow progenitors were suspended in a mixture containing 30 percent fetal calf serum (FCS) (Gibco), 1 percent bovine serum albumin (Sigma), $10^{-4}M$ mercaptoethanol (Sigma), penicillin, streptomycin, 0.9 percent methylcellulose, and serial dilutions of the conditioned medium or recombinant GM-CSF (protein concentration 100 μg/ml). Mo cell–conditioned medium (Mo-CM) prepared from the human Mo T-lymphoblast cell line was kindly provided by D. Golde and J. Gasson. The bone marrow progenitor cells were plated in duplicate at 0.75×10^4 to 1×10^4 cells per milliliter in flat-bottomed 24-well tissue culture plates (Linbro). Plates were incubated at 37°C in a high humidity 5 percent CO_2–95 percent air incubator. After 3 days, human urinary erythropoietin (Terry Fox Laboratories) was added to each well at a final concentration of 2 U/ml. Colonies were counted on day 14. Colony formation was almost absent unless Mo-CM or recombinant GM-CSF was added. This low background is the result of delayed addition of erythropoietin.
10. C. A. Westbrook *et al.*, *J. Biol. Chem.* **259**, 9992 (1984).
11. D. W. Golde, N. Bersch, S. G. Zuan, A. J. Lusis, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 593 (1980); J. C. Gasson *et al.*, *Nature (London)* **315**, 768 (1985).
12. Blood mononuclear cells from normal volunteers were separated on Ficoll-Paque and monocytes depleted by two sequential adherence procedures, the first for 3 hours and the second overnight. Nonadherent cells were incubated with sheep red blood cells treated with 2-aminoethylisothiouronium bromide and T-lymphocyte rosettes depleted by centrifugation through Ficoll-Paque. The nonadherent, T-lymphocyte depleted blood mononuclear cells were suspended at a final concentration of 5×10^4 per milliliter in methylcellulose culture mix (*9*) and human urinary erythropoietin (1 U/ml) added on day 0. The addition of erythropoietin on day 0 induces a background of between 10 and 20 erythroid colonies per 10^4 target cells plated.
13. K. Welte, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 1526 (1985).
14. D. Metcalf, G. R. Johnson, A. W. Burgess, *Blood* **55**, 183 (1980).
15. C. A. Sieff *et al.*, *J. Clin. Invest.*, in press.
16. S. G. Emerson *et al.*, *ibid.*, **76**, 1286 (1985).
17. D. C. Linch and D. G. Nathan *Nature (London)* **312**, 775 (1984).
18. J. Ritz, *Clin. Hematol.* **12**, 813 (1983); R. O'Reilly, *Blood* **62**, 941 (1983).
19. G. Hale *et al.*, *Blood* **62**, 873 (1983); P. C. L. Beverley, *Nature (London)* **287**, 332 (1980); R. F. Todd *et al.*, *J. Immunol.* **126**, 1435 (1981); J. D. Griffin *et al.*, *J. Clin. Invest.* **68**, 932 (1981); P. A. W. Edwards, *Biochem. Soc. Trans.* **8**, 334 (1980). Monoclonal antibodies were obtained from the following sources: Leu 1, Leu 5B, and Leu M1 from Becton-Dickinson; Campath and YTH 89.18 from H. Waldmann; TG1 from P. Beverley; Mo 1 and My 8 from J. Griffin; and LICR.LON.R18 from P. Edwards.

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