Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 4449–4452, June 1990
Cell Biology

Specific inhibition of interleukin 3 bioactivity by a monoclonal antibody reactive with hematopoietic progenitor cells

PETER D. EMANUEL*, STEPHEN C. PEIPER†, ZHANG CHEN‡, DE CHEN SHENG‡, AND KENNETH S. ZUCKERMAN*§

*Departments of Medicine and †Pathology and Comprehensive Cancer Center, University of Alabama at Birmingham, Birmingham, AL 35294; ‡Institute of Hematology, Chinese Academy of Medical Sciences, Tianjin, People's Republic of China

Communicated by Eugene P. Cronkite, April 2, 1990

ABSTRACT **HIM1, originally designated HI98, a murine monoclonal IgM antibody raised against human mononuclear cells, has been reported at the Fourth International Leukocyte Typing Workshop (called antibody M0141) to be the only one of 157 antibodies tested that inhibited binding of interleukin 3 (IL-3) to KG-1 human acute myelogenous leukemia cells and normal human monocytes. We have carried out detailed studies of the selective effect of HIM1 on IL-3-mediated stimulation of hematopoietic progenitors. Preincubation of normal human bone marrow mononuclear cells, depleted of adherent cells and T cells, with HIM1 antibody resulted in a dose-dependent inhibition of IL-3-mediated stimulation of both erythroid burst-forming units (maximum inhibition 55%) and granulocyte/macrophage colony-forming units (maximum inhibition 49%). HIM1 antibody had no effect on growth of erythroid colony-forming units in culture. In addition, preincubation of the cells with HIM1 antibody had no deleterious effect on granulocyte/macrophage colony-stimulating factor-induced growth of either erythroid bursts or granulocyte/macrophage colonies. To be certain that the HIM1 antibody did not react directly with IL-3 itself, we attempted to use immunodepletion to remove IL-3 that had been added to our culture medium. Although we were able to remove IL-3 bioactivity by immunodepletion with anti-IL-3 antibody bound to Sepharose beads, beads with attached HIM1 did not remove IL-3 activity from the medium. Polymorphonuclear neutrophils bind high levels of HIM1, although they have very few or no detectable IL-3 receptors. Therefore, this antibody appears to recognize a cell surface antigen that is critical for optimal IL-3 binding and bioactivity but is not the actual IL-3 receptor.**

Normal and leukemic hematopoietic cells express a repertoire of cell surface antigens that reflect their state of differentiation and maturation. Many of these antigens were first identified as epitopes recognized by monoclonal antibodies. Currently, at least 78 generic antibody specificities for human leukocytes are recognized. Approximately 13 of these are expressed preferentially by cells of the myeloid lineage, and all except 1 are glycoproteins (1). None of the available monoclonal antibodies produced against myeloid cells recognizes a membrane determinant that is involved in regulating the proliferation or differentiation of hematopoietic progenitors.

Several factors have been identified that influence hematopoiesis by binding to specific receptors on the cell surface. Of the colony-stimulating factors (CSFs) and cytokines known to stimulate hematopoiesis, receptors for macrophage (M-) CSF, granulocyte/macrophage (GM-) CSF, interleukin (IL-) 1, IL-2, IL-4, and IL-6 have been characterized and molecularly cloned (2–9). However, the receptors for IL-3 and granulocyte (G-) CSF have been incompletely characterized. HIM1 (originally designated HI98, and then as M0141 at the Fourth International Leukocyte Typing Workshop) was found to have the unique ability, among existing myeloid cell surface-reactive monoclonal antibodies, to inhibit binding of recombinant human IL-3 to human target cells (10).

Elucidating the mechanism(s) of IL-3 signaling is of particular importance due to the major stimulatory effects of this hemopoietin on multipotent and committed progenitors, as well as mature eosinophils, basophils, and macrophages (11, 12). The human IL-3 gene has been cloned and encodes a 152-amino acid protein, of which 19 amino acids are a cleaved signal peptide (13, 14).

In addition to its role as a regulator of early events in normal hematopoiesis, it is conceivable that IL-3 may have an autocrine role in leukemia. The WEHI-3B mouse acute myelogenous leukemia (AML) cell line has constitutive expression of IL-3 due to insertional gene activation (15). Recombinant human IL-3 stimulates the proliferation of AML cells in clonogenic assays (16) and has been found to enhance the growth of nonhematopoietic tumor cell lines *in vitro* (17). Constitutive expression of IL-3 has been achieved in murine hematopoietic progenitors by infecting stem cells with recombinant retroviruses containing sequences encoding IL-3. Animals reconstituted with these stem cells developed a myeloproliferative disorder leading to infiltration of viscera by myeloid cells and death within 4 weeks (18).

IL-3 binds to a high-affinity receptor that is expressed at low levels on the surface of murine and human bone marrow cells and their leukemic cell line counterparts. Preliminary chemical crosslinking studies have indicated that there are two predominant murine IL-3-binding proteins (65–70 kDa and 140 kDa) (19, 20), although affinity adsorption studies have revealed that several polypeptides may be involved in this interaction (19). There is also evidence that a hybrid IL-3/GM-CSF receptor may exist. Gesner *et al.* (21) showed that GM-CSF competed with IL-3 for binding to the KG-1 human AML cell line under conditions that prevent receptor internalization. Recently this group (22) has demonstrated that while there may be cross-competition for binding between IL-3 and GM-CSF, there are distinct binding proteins with approximate sizes of 69 and 93 kDa for IL-3 and GM-CSF, respectively. Kannourakis *et al.* (23) have data that support the distinct-receptor hypothesis but suggest that the cross-competition may occur due to association of the receptor complex through a common 85-kDa subunit.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: GM-CSF, granulocyte/macrophage colony-stimulating factor; IL, interleukin; AML, acute myelogenous leukemia; BFU-E, erythroid burst-forming unit(s); CFU-E, erythroid colony-forming unit(s); CFU-GM, granulocyte/macrophage colony-forming unit(s).

§To whom reprint requests should be addressed at: Division of Hematology/Oncology, 513 Tinsley Harrison Tower, University of Alabama at Birmingham, Birmingham, AL 35294.

We now characterize the biologic activity of a monoclonal antibody that will be helpful in elucidating further the mechanisms of IL-3 signaling. The HIM1 monoclonal antibody was produced by using a strategy to select for determinants preferentially expressed by primitive hematopoietic cells and monocytes. Mice were immunized with human blood mononuclear cells, a fraction enriched for lymphocytes, monocytes, and hematopoietic progenitor cells but depleted of granulocytes. HIM1, originally called HI98, was identified as an antibody that bound to myeloid cells but lacked reactivity with lymphocytes (24). Lewis *et al.* (10), in testing 157 myeloid cell antibodies for the Fourth International Leukocyte Typing Workshop, found that this antibody (called M0141 in their report) was the only one that inhibited IL-3 binding to the KG-1 human AML cell line (by a maximum of 50%) and to normal human blood monocytes (by a maximum of 86%, with a 200-fold molar excess of antibody). Neither this antibody nor any of the 156 others inhibited GM-CSF binding to the HL-60 human promyelocytic cell line, G-CSF binding to the U-937 human monocytic cell line, or IL-4 binding to KG-1 cells. To examine the potential physiologic significance of the observation that HIM1 blocked IL-3 binding, experiments were performed to determine the ability of HIM1 to alter the biologic activity of IL-3 in stimulating hematopoietic progenitor cell proliferation and differentiation *in vitro*.

MATERIALS AND METHODS

Hematopoietic Progenitor Cell Assays. With the approval of the Institutional Review Board of the University of Alabama at Birmingham and after donor consent, bone marrow aspirate was obtained from the posterior superior iliac spine of normal donors. After the aspirate was collected in preservative-free heparin, the mononuclear cell fraction was harvested by density centrifugation on Histopaque-1.077 separation medium (Sigma). Before use, the mononuclear cells were washed three times with Hanks' balanced salt solution (HBSS) containing 10% fetal bovine serum. They were then plated at 20×10^6 cells per 10 ml in a 100-mm tissue culture dish in HBSS with 13% AB serum and incubated overnight at 37°C. The nonadherent cells were transferred to a second plate for a subsequent 60-min adherence depletion. T lymphocytes were removed by panning with anti-Leu-1 and anti-Leu-5b antibodies (Becton Dickinson). The subsequent cell populations, depleted of adherent mononuclear phagocytes and T cells, were used in the assays described below. Cytospin preparations always revealed <4% contaminating monocytes. Plasma clot cultures were prepared as described (25). Recombinant human erythropoietin (Amgen Biologicals) was included at 1 unit/ml. Recombinant human GM-CSF (Genzyme) was used as a growth factor in some assays, at a final concentration of 100 units/ml. This concentration has been found in our lab to confer maximal or near-maximal stimulatory activity on erythroid burst-forming units (BFU-E) and granulocyte/macrophage colony-forming units (CFU-GM) (P.D.E. and K.S.Z., unpublished data). Recombinant human IL-3, provided by Steve Clark (Genetics Institute, Cambridge, MA) as culture supernatant from transfected COS cells had maximal activity in clonal CFU-GM and BFU-E assays at approximately 1:10,000 to 1:5000 dilution and was used at a final dilution of 1:10,000 in the hematopoietic progenitor cell cultures. The adherent cell-depleted, T-cell-depleted mononuclear cells were incubated in aliquots with various dilutions of the HIM1 antibody ranging from 1:100 to 1:100,000 (final culture concentration) for 1 hr at 4°C before the cultures were set up. An irrelevant IgM monoclonal antibody at a final dilution of 1:200 was employed as a control. Cultures were performed in triplicate with 10^5 cells per 0.3 ml of plasma clot culture and incubated for 7 days for erythroid CFU (CFU-E) counts and 12–14 days for BFU-E and CFU-GM counts. CFU-E-derived colonies contained 8–64 benzidine-positive cells. BFU-E-derived colonies contained >64 benzidine-positive cells and/or >2 subcolonies. CFU-GM-derived colonies contained >40 neutrophils and/or macrophages.

Immunodepletion of IL-3. To be certain that HIM1 antibody did not react directly with IL-3 itself, we used immunodepletion to determine whether HIM1 could bind to IL-3 and remove it from our culture medium. Protein G-Sepharose CL-4B beads (Pharmacia) were coated with goat antibody reactive against mouse IgG and IgM (Jackson Immunoresearch) on a rotary mixer for 2 hr at 4°C. After extensive washing, aliquots of these coated beads were incubated with either HIM1 antibody (final dilution 1:30), monoclonal murine anti-human IL-3 (Genzyme) (final dilution 1:6), or an irrelevant IgM monoclonal antibody (final dilution 1:30) for 2 hr on a rotary mixer at 4°C, followed by repeated washing and resuspension in phosphate-buffered saline. IL-3 was added at a final dilution of 1:10,000 in Iscove's medium and incubated with the antibody-coated beads for 2 hr at 4°C with gentle agitation. After microcentrifugation, the supernatant was removed and subjected to two more identical cycles of immunodepletion by immunoadsorption, using fresh antibody-coated beads each time. The final supernatants were placed into plasma clot cultures of adherent cell- and T-cell-depleted bone marrow mononuclear cells as described above. Residual IL-3 in the supernatants was determined by its ability to stimulate growth of BFU-E-derived colonies. To rule out nonspecific inhibition of colony growth by the supernatants from which IL-3 had been immunodepleted, IL-3 was added to cultures in the presence of immunodepleted supernatant and found to have the expected stimulatory effect on BFU-E growth.

RESULTS

The purpose of our studies was to determine whether the cell surface structure recognized by the HIM1 antibody plays an important role in the biologic activity of IL-3. Therefore, we systematically examined whether preincubation of bone marrow cells with HIM1 antibody selectively inhibited the ability of IL-3 to stimulate growth of hematopoietic progenitor cells. The results are the summary of seven separate experiments, although not all antibody concentrations were used in every experiment. Preincubation of the cells with HIM1 antibody resulted in a dose-dependent inhibition of IL-3-mediated stimulation of BFU-E growth *in vitro*, achieving maximal inhibition (55%) at an HIM1 dilution of 1:200 (Fig. 1). The specificity of the effect of IL-3 was demonstrated by the absence of an alteration in BFU-E growth stimulated by GM-CSF (Fig. 1). An irrelevant monoclonal IgM antibody also was tested in each experiment at a dilution of 1:200 and consistently resulted in <5% inhibition of either IL-3- or GM-CSF-stimulated BFU-E growth. Likewise, preincubation of the cells with the HIM1 antibody resulted in a dose-dependent inhibition of IL-3-stimulated CFU-GM growth, with maximal inhibition (49%) at a 1:200 dilution of the antibody (Fig. 2). As was the case with BFU-E, HIM1 had no inhibitory effect on CFU-GM growth stimulated by GM-CSF. The irrelevant control antibody also did not inhibit CFU-GM growth. Preincubation of the cells with the HIM1 antibody had no effect on CFU-E growth in either the IL-3-stimulated cultures or the GM-CSF-stimulated cultures (Fig. 3).

To confirm that the biologic effects of the HIM1 antibody were the result of interaction with a cell surface molecule involved with the binding and stimulatory effect of IL-3 on hematopoietic progenitor cells, and that HIM1 did not exert its inhibitory effects merely by binding directly to IL-3,

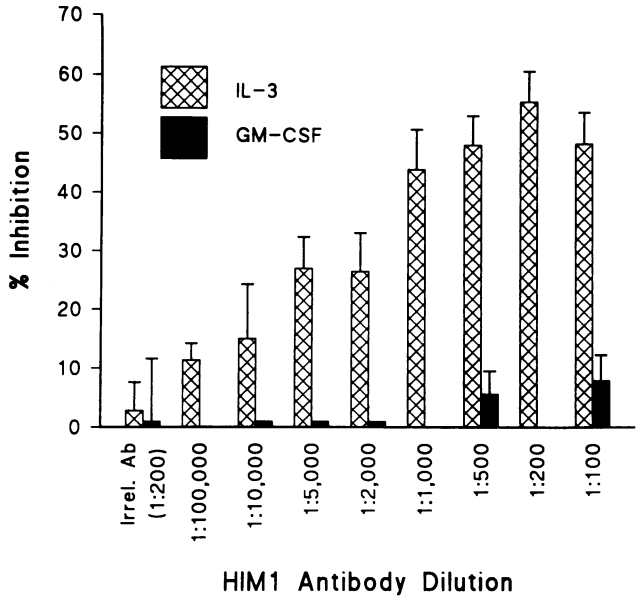

FIG. 1. Inhibitory effect of HIM1 antibody on BFU-E growth *in vitro*. Adherent cell- and T-cell-depleted peripheral blood mononuclear cells were preincubated with the indicated final dilutions of HIM1 antibody or irrelevant control antibody (Irrel. Ab) for 1 hr at 4°C. Then, 5×10^4 cells were cultured in 0.3-ml plasma clots for 14 days in the presence of erythropoietin (1 unit/ml) and either IL-3 (1:10,000 dilution of transfected COS cell supernatant) or GM-CSF (100 units/ml). The number of BFU-E observed in the absence of added IL-3 or GM-CSF always was <3 colonies per culture. Without antibody preincubation, IL-3 stimulated 70 ± 4 and GM-CSF elicited 42 ± 5 BFU-E per 5×10^4 cells (mean ± SEM, $n = 7$).

immunoadsorption experiments were performed. As expected, we were able specifically to deplete IL-3 from culture medium by three sequential incubations with Sepharose beads coated with monoclonal anti-IL-3 antibody. Medium from which IL-3 had been immunoadsorbed with the anti-

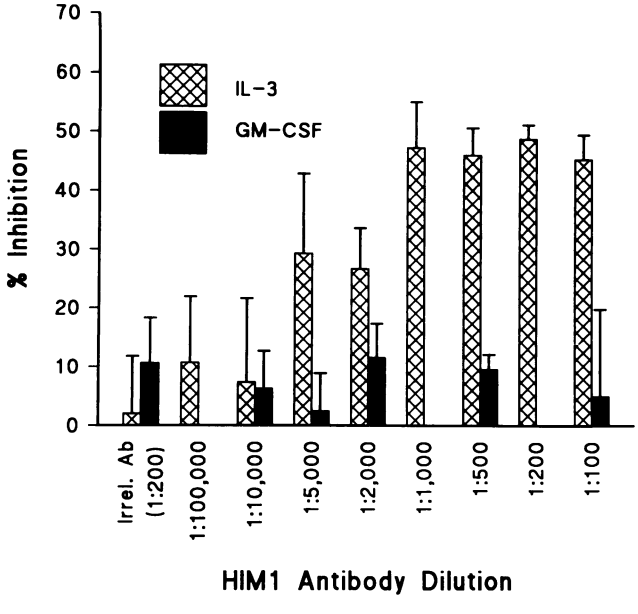

FIG. 2. Inhibitory effect of HIM1 antibody on CFU-GM growth *in vitro*. Incubations and culture conditions were as described in the legend of Fig. 1 and in *Materials and Methods*. The number of CFU-GM observed in the absence of added IL-3 or GM-CSF always was <5 colonies per culture. Without antibody preincubation, IL-3 stimulated 34 ± 4 and GM-CSF elicited 38 ± 3 CFU-GM per 5×10^4 cells (mean ± SEM, $n = 7$).

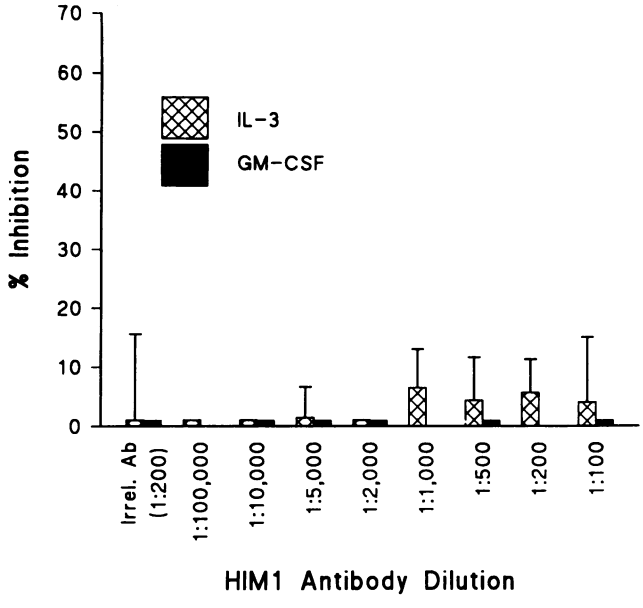

FIG. 3. Lack of inhibitory effect of HIM1 antibody on CFU-E growth *in vitro*. Incubations and culture conditions were as described for Fig. 1. (Note: CFU-E were enumerated after 7 days in culture.) In the absence of added IL-3 or GM-CSF the number of CFU-E observed was 67 ± 13 (mean ± SEM, $n = 7$). Addition of IL-3 resulted in 143 ± 12, and GM-CSF stimulated 113 ± 34 CFU-E per culture (mean ± SEM, $n = 7$).

IL-3-coated beads supported only 8% of control BFU-E growth (Table 1). The BFU-E-stimulating activity in these supernatants could be restored completely by the addition of IL-3, indicating that the effect was specific (data not shown). In contrast, IL-3-containing medium immunoadsorbed with similar beads coated with HIM1 antibody or irrelevant antibody supported BFU-E growth that was 72% and 83%, respectively, of that obtained with control IL-3-containing medium (Table 1), indicating that HIM1 antibody does not exert its inhibitory effect by binding to IL-3 itself.

DISCUSSION

We have established that the HIM1 monoclonal IgM antibody, in addition to its previously demonstrated inhibitory effect on IL-3 binding to hematopoietic cells, also selectively inhibits IL-3-stimulated proliferation and differentiation of these progenitors. Our data showing selective 55% maximal inhibition of BFU-E and 49% inhibition of CFU-GM growth stimulated by IL-3, but no effect on colony growth stimulated by GM-CSF, agree with the results of IL-3 and GM-CSF binding studies done by Lewis *et al.* (10). In studies to be published elsewhere, we have obtained data demonstrating that HIM1 binds strongly to mature granulocytes (S.C.P., P.D.E., and K.S.Z., unpublished data), which neither bind nor are activated by IL-3 (26, 27). Therefore, it does not appear that the HIM1 antibody recognizes an epitope on the IL-3 receptor, but that the antigen recognized is a critical accessory component that is important for optimal IL-3

Table 1. Immunodepletion of IL-3

Antibody used for immunodepletion of IL-3-containing supernatant	BFU-E, mean ± SEM (% of control)
None	99 ± 8 (100)
Irrelevant antibody	82 ± 11 (83)
HIM1 antibody	71 ± 5 (72)
Anti-IL-3 antibody	8 ± 6 (8)

stimulation of BFU-E and CFU-GM growth. These results lead us to speculate that one of the following is operative: (*i*) there are at least two distinct IL-3-recognition molecules on myeloid cells, and IL-3 binding to both (or all) of these sites is required for optimal stimulation of hematopoietic progenitor cells; (*ii*) HIM1 antibody recognizes a cell surface protein that combines with the IL-3/IL-3 receptor complex, enhances binding of IL-3 to its receptor and, hence, enhances the ability of IL-3 to activate hematopoietic progenitor cells; or (*iii*) HIM1 binds to a surface antigen that is physically associated with the IL-3 receptor and sterically inhibits association of IL-3 with its receptor. There is ample precedent for the second possibility, in that at least three other cytokines have multisubunit receptor complexes. Interaction of IL-6 with its 80-kDa binding protein (8) leads to association with a 130-kDa accessory protein to form an activated receptor complex (9). The 130-kDa accessory protein is presumed to function as a signal-transducing subunit. The high-affinity IL-2 receptor is a heterodimer composed of 55-kDa and 70- to 75-kDa subunits (6). These two polypeptides may be expressed dyssynchronously to form low- and intermediate-affinity IL-2 receptors. The genes encoding the erythropoietin receptor subunits have been molecularly cloned and the receptor complex has both low- and high-affinity forms (28, 29). D'Andrea *et al.* (30) reported several structural features shared by the erythropoietin receptor and the IL-2 receptor β chain and have proposed that they belong to a new receptor family.

Our data demonstrate that HIM1 monoclonal antibody recognizes a cell surface molecule involved in the binding to and stimulation of hematopoietic progenitors by IL-3. Further biochemical and genetic characterization of the antigen it recognizes should enhance our understanding of the mechanism of IL-3 binding and signaling at the plasma membrane.

This work was supported by U.S. Public Health Service Grants DK07488, DK08408, DK41935, CA13148, and CA01013.

1. *Leukocyte Typing IV* (1989), ed. Knapp, W. (Oxford Univ. Press, Oxford), 747–748.
2. Sherr, C. J., Rettenmier, C. W., Sacca, R., Roussel, M. F., Look, A. T. & Stanley, E. R. (1985) *Cell* **41**, 665–676.
3. Gearing, D. P., King, J. A., Gough, N. M. & Nicola, N. A. (1989) *EMBO J.* **12**, 3667–3676.
4. Bomsztyk, K., Sims, J. E., Stanton, T. H., Slack, J., McMahan, C. J., Valentine, M. A. & Dower, S. K. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 8034–8038.
5. Chizzonite, R., Truitt, T., Kilian, P. L., Stern, A. S., Nunes, P., Parker, K. P., Kaffka, K. L., Chua, A. O., Lugg, D. K. & Gubler, U. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 8029–8033.
6. Hatakeyama, M., Tsudo, M., Minamoto, S., Kono, T., Doi, T., Miyata, T., Miyasaka, M. & Taniguchi, T. (1989) *Science* **244**, 551–556.
7. Mosley, B., Beckmann, M. P., March, C. J., Idzerda, R. L., Gimpel, S. D., VandenBos, T., Friend, D., Alpert, A., Anderson, D., Jackson, J., Wignall, J. M., Smith, C., Gallis, B., Sims, J. E., Urdal, D., Widmer, M. B., Cosman, D. & Park, L. S. (1989) *Cell* **59**, 335–348.
8. Yamasaki, K., Taga, T., Hirata, Y., Yawata, H., Kawanishi, Y., Seed, B., Taniguchi, T., Hirano, T. & Kishimoto, T. (1988) *Science* **241**, 825–828.
9. Taga, T., Hibi, M., Hirata, Y., Yamasaki, K., Yasukawa, K., Matsuda, T., Hirano, T. & Kishimoto, T. (1989) *Cell* **58**, 573–581.
10. Lewis, A. J., Urdal, D. L. & Park, L. S. (1989) *Tissue Antigens* **33**, 227.
11. Ihle, J. N., Keller, J., Oroszlan, S., Henderson, L. E., Copeland, T. D., Fitch, F., Prystowsky, M. B., Goldwasser, E., Schrader, J. W., Palaszynski, E., Dy, M. & Lebel, B. (1983) *J. Immunol.* **131**, 282–287.
12. Schrader, J. W. (1986) *Annu. Rev. Immunol.* **4**, 205–230.
13. Otsuka, T., Miyajima, A., Brown, N., Otsu, K., Abrams, J., Saeland, S., Caux, C., DeWaal Malefijt, R., De Vries, J., Meyerson, P., Yokota, K., Gemmel, L., Rennick, D., Lee, F., Arai, N., Arai, K. & Yokota, T. (1988) *J. Immunol.* **140**, 2288–2295.
14. Yang, Y.-C., Ciarletta, A. B., Temple, P. A., Chung, M. P., Kovacic, S., Witek-Giannotti, J. S., Leary, A. C., Kriz, R., Donahue, R. E., Wong, G. G. & Clark, S. C. (1986) *Cell* **47**, 3–10.
15. Ymer, S., Tucker, W. Q. J., Sanderson, C. J., Hapel, A. J., Campbell, H. D. & Young, I. G. (1985) *Nature (London)* **317**, 255–258.
16. Delwel, R., Dorssers, L., Touw, I., Wagemaker, G. & Löwenberg, B. (1987) *Blood* **70**, 333–336.
17. Berdel, W. E., Danhauser-Riedl, S., Steinhawer, G. & Winton, E. F. (1989) *Blood* **73**, 80–83.
18. Chang, J. M., Metcalf, D., Lang, R. A., Gonda, T. J. & Johnson, G. R. (1989) *Blood* **73**, 1487–1497.
19. Stevens, D. A., Schrews, J., Ihle, J. N. & May, W. S. (1989) *Exp. Hematol.* **17**, 648.
20. Murthy, S. C., Mui, A. L.-F. & Krystal, G. (1990) *Exp. Hematol.* **18**, 11–17.
21. Gesner, T. G., Mufson, R. A., Norton, C. R., Turner, K. J., Yang, Y.-C. & Clark, S. C. (1988) *J. Cell. Physiol.* **136**, 493–499.
22. Gesner, T., Mufson, R. A., Turner, K. J. & Clark, S. C. (1989) *Blood* **74**, 2652–2656.
23. Kannourakis, G., Jubinsky, P. & Sieff, C. A. (1989) *Blood* **74**, Suppl. 1, 567.
24. Sheng, D. C. & Chen, Z. (1989) *J. Chin. Hematol.* **10**, 350–353.
25. Zuckerman, K. S., Bagby, G. C., Jr., McCall, E., Sparks, B., Wells, J., Patel, V. & Goodrum, D. (1985) *J. Clin. Invest.* **75**, 722–725.
26. Lopez, A. F., Dyson, P. G., To, L. B., Elliott, M. J., Milton, S. E., Russell, J. A., Juttner, C. A., Yang, Y.-C., Clark, S. C. & Vadas, M. A. (1988) *Blood* **72**, 1797–1804.
27. Nicola, N. A. & Metcalf, D. (1986) *J. Cell. Physiol.* **128**, 180–188.
28. D'Andrea, A. D., Lodish, H. F. & Wang, G. G. (1989) *Cell* **57**, 277–285.
29. Sawyer, S. T., Krantz, S. B. & Sawada, K. (1989) *Blood* **74**, 103–109.
30. D'Andrea, A. D., Fasman, G. D. & Lodish, H. F. (1989) *Cell* **58**, 1023–1024.