

# Selection of Lineage-restricted Cell Lines Immortalized at Different Stages of Hematopoietic Differentiation from the Murine Cell Line 32D

Giovanni Migliaccio,\* Anna Rita Migliaccio,\* Brent L. Kreider,‡ Giovanni Rovera,‡  
and John W. Adamson\*

\* Division of Hematology, Department of Medicine, University of Washington, Seattle, Washington 98195; and

‡ The Wistar Institute, Philadelphia, Pennsylvania 19104

**Abstract.** Erythropoietin (Epo), granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor- (G-CSF) dependent cell lines have been derived from the murine hematopoietic cell line 32D with a selection strategy involving the culture of the cells in FBS-deprived medium supplemented only with pure recombinant Epo, GM-CSF, or G-CSF. The cells retain the diploid karyotype of the original 32D clone, do not grow in the absence of exogenous growth factor, and do not induce tumors when injected into syngeneic recipients. The morphology of the Epo-dependent cell lines (32D Epo1, -2, and -3) was heterogeneous and evolved with passage. The percent of differentiated cells also was a function of the cell line investigated. Benzidine-positive cells ranged from 1–2% (32D Epo3) to 50–60% (32D Epo1). These erythroid cells expressed carbonic anhydrase I and/or globin mRNA but not carbonic anhydrase II. The GM-CSF- and G-CSF-de-

pendent cell lines had predominantly the morphology of undifferentiated myeloblasts or metamyelocytes, respectively. The GM-CSF-dependent cell lines were sensitive to either GM-CSF or interleukin-3 (IL-3) but did not respond to G-CSF. The G-CSF-dependent cell lines grew to a limited extent in IL-3 but did not respond to GM-CSF. These results indicate that the cell line 32D, originally described as predominantly a basophil/mast cell line, has retained the capacity to give rise to cells which proliferate and differentiate in response to Epo, GM-CSF, and/or G-CSF. These cells represent the first nontransformed cell lines which can be maintained in growth factors other than IL-3 and which differentiate in the presence of physiologic signals. As such, they may represent a model to study the molecular mechanisms underlying the process of hematopoietic differentiation, as well as sensitive targets for bioassays of specific growth factors.

**32D** is a diploid interleukin- (IL)-3-dependent cell line established from long term marrow cultures of C3He/J mice injected with the Friend murine leukemia virus (Greenberger et al., 1980a; Greenberger et al., 1983). The cells express proteins immunologically related to the gag and env proteins of the family of ecotropic viruses (Ruscetti, S., personal communication) and release into the medium detectable levels of reverse transcriptase (Kreider, B., and G. Rovera, unpublished observation). However, the cells do not release detectable amounts of infective virus (Greenberger et al., 1980b, 1983), do not induce tumors when injected into histocompatible recipients (Greenberger et al., 1980b, 1983) and give rise to spontaneously transformed and growth factor-independent cells at extremely low frequency. For these reasons, it is unlikely these cells are actually transformed by Friend murine leukemia virus.

*1. Abbreviations used in this paper:* Epo, erythropoietin; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin.

Thus, 32D is considered to be a population of immortalized "normal" hematopoietic progenitors and has been used to study the process of control of normal hematopoiesis and how this control is altered during leukemogenesis. The two most commonly used versions of this cell line have been named 32D cl 23 and 32D cl 3.

In contrast to the cell line B6SutA, which may be induced to form colonies composed of erythroid cells, neutrophil-granulocytes, and basophil-mast cells, 32D was originally described to form colonies composed only of basophil/mast cells (Greenberger et al., 1983). This result suggested that the cells were already restricted in their differentiative potential. Metcalf (1985) reported that IL-3, but not granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), or macrophage colony-stimulating factor had the capacity to support survival and colony formation of 32D cl 3. On this basis, 32D cl 23 growth was used to discriminate between the presence of IL-3 and GM-CSF in studies characterizing the cytokines

produced by murine stromal cells (Naparstek et al., 1986; Gualtieri et al., 1987) or activated cloned T-lymphocytes (Kelso, 1986). However, Watson et al. (1986) purified human G-CSF to homogeneity from medium conditioned by the bladder carcinoma cell line 5637 using a bioassay involving the induction of [<sup>3</sup>H]thymidine uptake of the 32D cl 23 cell line, and Valtieri et al. (1987) reported that pure G-CSF induces DNA synthesis and neutrophil differentiation of 32D cl 3. Furthermore, 32D cl 3, passed several times in medium containing G-CSF and IL-3 (32D cl 3(G)), acquires the capacity to proliferate also in response to GM-CSF (Rovera et al., in press). However, both 32D cl 3 and 32D cl 3(G) require IL-3 for long term survival (Valtieri et al., 1987; Rovera et al., 1989). In a similar study, Le Gros et al. (1987) reported that, although 32D cl 23 cells bear only low affinity IL-2 receptors and do not proliferate in response to IL-2 (Koyasu et al., 1986), they may acquire high affinity IL-2 receptors and the capacity to proliferate in the presence of IL-2 in 1–2 mo of continuous passage in cultures containing IL-2 and IL-3.

The presence of receptors for growth factors other than IL-3 seems to be a common feature of IL-3-dependent cell lines. As examples, low affinity IL-2 receptors have been found not only on 32D cl 23, but also on FDCP-1 cell lines (Koyasu et al., 1986); high affinity IL-4 receptors have been found on 32D, FDCP-1, NFS-60 (Park et al., 1987; Lowenthal et al., 1988), and DA-1 (Ohara and Paul, 1987) cell lines; and high affinity erythropoietin (Epo) receptors have been found on IC-2, DA-1, and FDCP-2 cell lines (Tsao et al., 1988).

The studies of receptor expression and the possibility of conditioning the 32D cell line to respond to other growth factors suggest that cells responding to growth factors other than IL-3 are normally generated in IL-3-dependent cell lines although at a very low frequency. This low frequency might not permit the further expansion of these clones in cultures supplemented only with IL-3. Therefore, we devised a selection strategy to isolate clones of 32D cells which proliferate in response to growth factors other than IL-3. This strategy involves the culture of 32D cl 3 or cl 3(G) in FBS-deprived cultures to which pure recombinant Epo, GM-CSF, or G-CSF has been added. With this approach, Epo-, GM-CSF-, or G-CSF-responsive cell lines were repeatedly isolated. We report here the characterization of three Epo-responsive, two GM-CSF-responsive, and two G-CSF-responsive cell lines.

## Materials and Methods

### Cell Lines

The 32D cl 3 (I) and 32D cl 3(G), that was conditioned to respond to G-CSF by repeated short treatments with G-CSF (Rovera et al., 1989) cell lines were maintained by bi-weekly passage in McCoy's medium (Gibco Laboratories, Grand Island, NY) modified as described (Greenberger et al., 1979) and supplemented with antibiotics, L-glutamine and pyruvic acid (1% [vol/vol]; Gibco Laboratories) and horse serum (10% [vol/vol]; HyClone Laboratories, Logan, UT). Conditioned medium from phytohemagglutinin-stimulated LBRM 33 cells (Gillis et al., 1980) (3% [vol/vol]) was added as a source of IL-3. The cell lines were periodically tested for the presence of mycoplasma contamination with Mycoplasma T.C.II (Gen-probe, San Diego, CA) and were mycoplasma free.

### Serum-deprived Cultures

The FBS was replaced by a mixture of nutrients composed of BSA (2 ×

10<sup>-4</sup> M), BSA-adsorbed cholesterol (12 μg/ml) and soybean lecithin (36 μg/ml), iron-saturated human transferrin (9 × 10<sup>-6</sup> M), bovine insulin (1.7 × 10<sup>-6</sup> M), nucleosides (10 μg/ml each), sodium pyruvate (10<sup>-4</sup> M), and L-glutamine (2 × 10<sup>-3</sup> M) as previously reported (Migliaccio and Migliaccio, 1987a,b). All the reagents were from Sigma Chemical Co. (St. Louis, MO) and prepared as described (Migliaccio and Migliaccio, 1987a).

### Colony Assay

The capacity of the cell lines to form colonies in response to the different growth factors was assessed in semisolid medium containing the following components in Iscove's modified Dulbecco's medium: methylcellulose (0.8% [wt/vol], final concentration), β-mercaptoethanol (7.5 × 10<sup>-5</sup> M), and the mixture of components described above which replaced the FBS. Colonies containing ≥5 × 10<sup>2</sup> cells per clone were scored at day 8 of culture.

### Hematopoietic Growth Factors

CM of PHA-stimulated LBRM 33 cells was prepared under serum-deprived conditions as described (Migliaccio and Migliaccio, 1987b). The growth factors studied included pure recombinant murine IL-3 (Metcalf et al., 1986a) and murine GM-CSF (Metcalf et al., 1986b) (provided by J. J. Mermod; Glaxo, Basel, Switzerland), and pure recombinant human Epo (Egrie et al., 1985) and human G-CSF (Souza et al., 1986) (Amgen, Thousand Oaks, CA). Unless otherwise stated, PHA-LBRM 33 CM was used at a concentration of 3% (vol/vol). The growth factors were used at concentrations of 10 U/ml for IL-3, 1 ng/ml for GM-CSF, 10<sup>3</sup> U/ml for G-CSF, and 5 U/ml for Epo. These concentrations have been shown previously to be maximally effective in promoting colony formation by purified murine progenitor cells under serum-deprived conditions (Migliaccio et al., 1988; Migliaccio et al., 1989).

### Cytochemistry

Cytopreparations of cells growing in log phase were stained with May-Grunwald/Giemsa or with staining specific for myeloperoxidase, chloroacetate esterase, or nonspecific esterase. All the reagents were obtained from Sigma Chemical Co. and used as recommended by the manufacturer. In some cases, the cells were benzidine stained to determine the presence of hemoglobin (Orkin et al., 1975).

### Northern Analysis

Total RNA was extracted from cells (5–10 × 10<sup>7</sup>) growing in log phase by the guanidinium-isothiocyanate method (Chirgwin et al., 1979), separated by electrophoresis on a 1.0% agarose/formaldehyde gel and transferred to nylon membranes (Zetabind, Cuno Laboratory Products, Meridian, CT) by Northern blotting (Thomas, 1980). The membranes were hybridized in the presence of 50% formamide and 20% dextran sulphate to 10<sup>7</sup> dpm of DNA probe labeled by nick-translation (Bethesda Research Laboratories, Gaithersburg, MD) or random oligonucleotide priming (Amersham International, Amersham, UK) to a specific activity of 4–8 × 10<sup>8</sup> dpm/mg, and washed at high stringency (0.1 × SSC, 0.1% SDS at 65°C) after established procedures (Thomas, 1980).

The β-globin probe (Nudel et al., 1977) used was the recombinant plasmid pM G, containing the cDNA of mouse β-major globin. The β-globin probe and the carbonic anhydrase I (Fraser and Curtis, 1986) and II (Curtis, 1983) probes were kindly provided by Dr. P. Curtis (Wistar Institute, Philadelphia, PA).

## Results

### Selection of Epo-, GM-CSF-, and G-CSF-dependent Cell Lines from the 32D cl 3 or 32D cl 3(G)

32D cl 3 and cl 3(G) form colonies with a 21–27% cloning efficiency in FBS-deprived cultures supplemented with PHA-LBRM 33 CM or IL-3 (Table I). Both cell lines also form colonies in cultures stimulated with GM-CSF or Epo although at a low frequency. 32D cl 3(G), but not 32D cl 3, formed colonies in the presence of G-CSF. To isolate the cells which can respond to Epo, GM-CSF, or G-CSF, 2.5 ×

**Table I. Cloning Efficiency of the Various Cell Lines Cultured in the Presence (FBS<sup>+</sup>) or Absence (FBS<sup>-</sup>) of Fetal Bovine Serum and with Various Growth Factors**

Cell line		Stimulus (clones per 10 <sup>4</sup> cells*)					
		None	LBRM 33 CM	IL-3	GM-CSF	G-CSF	Epo
32D cl 3	FBS <sup>+</sup>	0	2,890 ± 290	1,840 ± 270	4 ± 4	0	2 ± 1
	FBS <sup>-</sup>	0	2,780 ± 560	2,180 ± 390	87 ± 56	0	8 ± 4
32D Epo1	FBS <sup>+</sup>	0	22 ± 11	112 ± 40	0	0	720 ± 74
	FBS <sup>-</sup>	0	91 ± 27	110 ± 28	0	0	346 ± 55
32D Epo2	FBS <sup>+</sup>	0	250 ± 40	635 ± 15	0	0	1,180 ± 50
	FBS <sup>-</sup>	0	695 ± 53	1,080 ± 190	7 ± 2	11 ± 5	1,800 ± 210
32D Epo3	FBS <sup>+</sup>	0	502 ± 104	201 ± 13	0	0	0
	FBS <sup>-</sup>	0	789 ± 245	389 ± 93	0	0	1,310 ± 370
32D GM1	FBS <sup>+</sup>	0	1,920 ± 230	1,590 ± 220	1,750 ± 200	0	0
	FBS <sup>-</sup>	0	2,050 ± 400	1,230 ± 330	1,160 ± 66	1 ± 1	1 ± 1
32D GM2	FBS <sup>+</sup>	0	1,680 ± 160	1,710 ± 190	1,720 ± 180	0	0
	FBS <sup>-</sup>	0	—	—	—	—	—
32D cl 3(G)	FBS <sup>+</sup>	—	3,100 ± 620	2,900 ± 300	54 ± 22	25 ± 10	0.01 ± 0.01
	FBS <sup>-</sup>	0	2,130 ± 560	2,360 ± 320	19 ± 6	18 ± 6	2 ± 1
32D G1	FBS <sup>+</sup>	0	4 ± 1	5 ± 1	0	0	0
	FBS <sup>-</sup>	0	1,220 ± 130	583 ± 84 <sup>‡</sup>	0	910 ± 98	0
32D G2	FBS <sup>+</sup>	0	0	3 ± 1	0	0	0
	FBS <sup>-</sup>	0	1,250 ± 93	816 ± 69 <sup>‡</sup>	0	798 ± 73	0

\* PHA-LBRM 33 CM was used at a concentration of 3% (vol/vol). The growth factors were used at concentrations of 10 U/ml for IL-3, 1 ng/ml for GM-CSF, 10<sup>3</sup> U/ml for G-CSF, and 5 U/ml for Epo. Cells were cultured at a concentration of 10<sup>3</sup>, 10<sup>4</sup>, or 10<sup>5</sup> cells/ml. The results were pooled and normalized to 10<sup>4</sup> cells/ml. The results represent the means (± SD) of three experiments performed in duplicate.

<sup>‡</sup> The colonies in these cultures were smaller (≤5 × 10<sup>2</sup> cells) and mainly composed of dead cells.

10<sup>4</sup> 32D cl 3 or cl 3(G) cells were cultured in wells containing 1 ml of FBS-deprived medium supplemented with Epo (1 U/ml), GM-CSF (1 ng/ml), or G-CSF (10<sup>3</sup> U/ml). After 1–2 wk of incubation, virtually all the cells were dead, regardless of the culture conditions. However, signs of cell proliferation were observed between the third and fourth week of incubation. At the end of the fourth week, the wells in which cell proliferation was observed were separately transferred into flasks containing 10 ml of FBS-deprived medium supplemented with the same growth factor initially used in the well cultures. The cells were passed regularly and those which survived for a least eight passages (>1 mo) have been considered lines. A total of six selections were carried out and the results are summarized in Table II. In general, all transfers from microwells to flasks have continued to grow. The only exception have been Epo-dependent cells derived from 32D cl 3(G). When these cells were transferred into flasks, they died after three passages. Three Epo-dependent (32D Epo1, -2, and -3) and two GM-CSF-depend-

ent (32D GM1 and -2) cell lines have been derived from 32D cl 3 with this process. Two GM-CSF-dependent and four G-CSF-dependent cell lines have been derived from 32D cl 3(G). Of these cell lines, only two of the G-CSF-dependent (32D G1 and 2) will be described in detail here. The morphology of some of the cell lines is shown in Fig. 1. The frequency of the cells in the original population which gave rise to these cell lines is calculated to be approximately one in 10<sup>5</sup> cells. We have been unable to obtain G-CSF-dependent cell lines from 32D cl 3 or Epo-dependent cell lines from 32D cl 3(G).

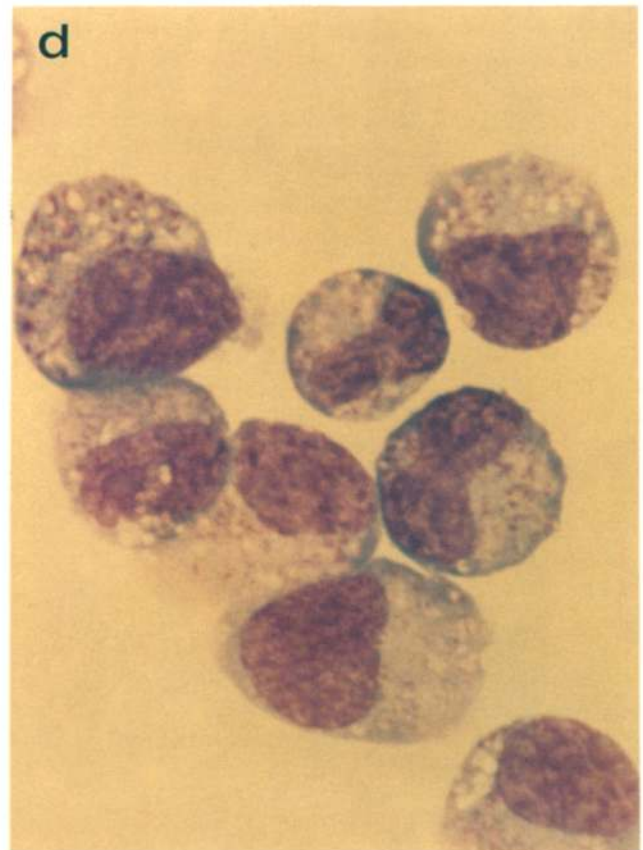
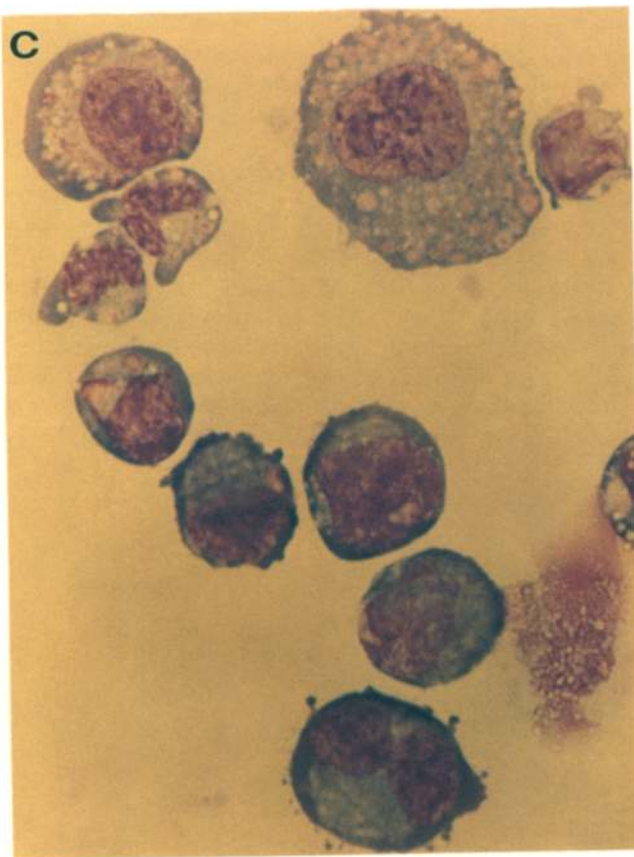
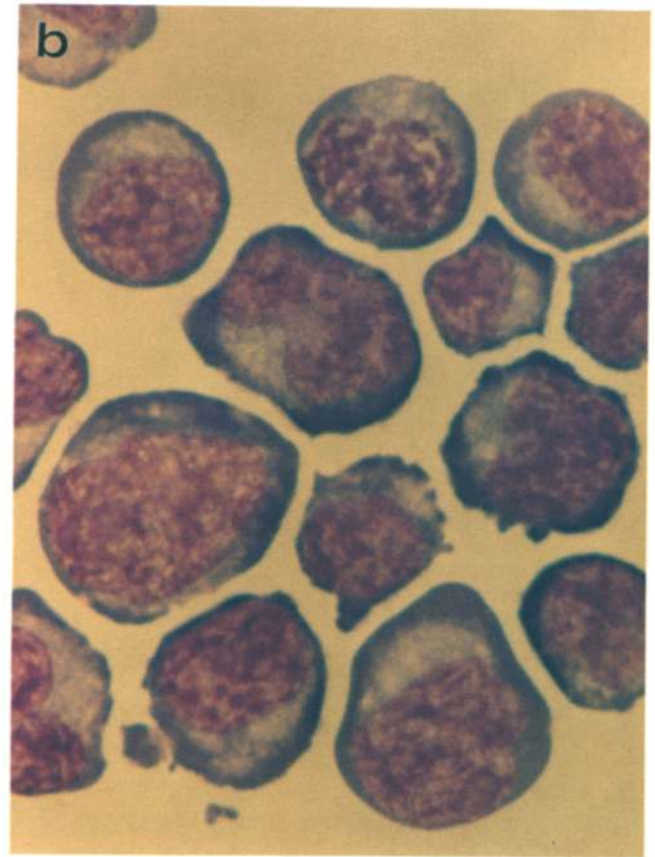
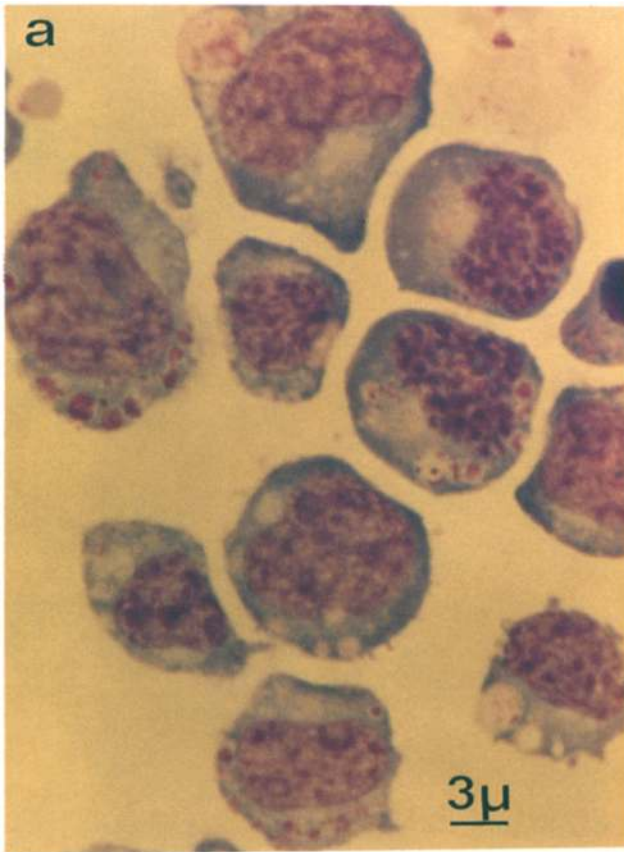
The cell lines derived from 32D cells failed to grow in the absence of growth factor (Table I).

The karyotype analysis of the derived cell lines, as well as of the original 32D cl 3 and cl 3(G), was performed according to standard methods. The 32D cl 3 was composed of two populations, one aneuploid (cell chromosome No. 38,X[-Y] [3% of the cells], No. 39,X[-Y] [90% of the cells] or No. 40 [XY] [6% of the cells]; modal chromosome No. 39,X[-Y])

**Table II. Selection of Cell Lines**

	32D cl 3				32D cl 3(G)			
	No stimulus	Epo	GM-CSF	G-CSF	No stimulus	Epo	GM-CSF	G-CSF
Total wells analyzed	10	10	8	10	10	18	6	12
Wells in which cell proliferation was detected and the cells amplified in flasks	0	3	2	0	0	10	2	4
Total flasks analyzed	—	3	2	—	—	10	2	4
Cell lines established	—	3	2	—	—	0	2	4

Six (two for the 32D cl 3 and four for the 32D cl 3[G]) experiments are reported. Epo, GM-CSF, or G-CSF were added at a concentration of 1 U/ml, 1 ng/ml, and 10<sup>3</sup> U/ml, respectively.



**Figure 1.** Morphology of the original 32D cl 3 cell line (a) and of Epo-dependent (32D Epo1)(b), GM-CSF-dependent (32D GM1)(c), and G-CSF-dependent (32D G1)(d) cell lines. Wright-Giemsa staining. The cells were obtained from cultures during the log phase of growth.

**Table III. Cytochemical Analysis of the Various Cell Lines\***

Cell line	Benzidine	Myeloperoxidase	Chloroacetate esterase	Nonspecific esterase
32D cl 3	0	1%	4%	0
32D cl 3(G)	0	1%	17%	1%
32D Epo1	50-60%	0	0	1%
32D Epo2	5-10%	0	0	1%
32D Epo3	1-2%	0	0	1%
32D GM1	0	1%	2%	13%
32D GM2	0	2%	1%	14%
32D G1	0	1%	44%	21%
32D G2	0	2%	29%	23%

\* All the cytochemical analyses were done on cells obtained at saturation density. 32D cl 3 and cl 3(G) were cultured in the presence of PHA-LBRM 33 CM; 32D Epo1, -2, and -3 were cultured in the presence of Epo; 32D GM1 and -2 were cultured in the presence of GM-CSF; and 32D G1 and -2 were cultured in the presence of G-CSF.

and the other polyploid (modal chromosome No. 78+). The ratio between the two populations was ~1:1. The 32D cl 3(G) cell line had a normal diploid male mouse chromosome complement (Valtieri et al., 1987). Both cell lines expressed glucose-6-phosphate dehydrogenase, nucleoside phosphorylase, malic dehydrogenase, and lactic dehydrogenase isoenzymes whose electrophoretic mobilities corresponded to the murine isotypes. All the cell lines isolated had a normal diploid male mouse chromosome complement, the modal number being 39,X[-Y]/40,XY. The frequency of aneuploid cells missing the Y chromosome was 30-50%, depending on the particular cell line.

To test the capability of the subclones derived from 32D to give rise to tumors, 10-12-wk-old C3H/HeJ female mice (The Jackson Laboratory, Bar Harbor, ME) were injected intravenously either with medium alone or with medium plus 32D Epo1, Epo2, GM1, or GM2 cells ( $10^7$  cells/mouse, five mice in each group). 11 mo later, 100% of the mice that received medium alone were dead while 100% of the mice that were injected with 32D Epo2, 75% of the mice that were injected with 32D GM1 or GM2, and 50% of the mice that were injected with 32D Epo1, were still alive. The majority of the animals died between 9 and 11 mo from the time of injection. None of the animals showed evidence of solid tumors or leukemia. The living animals were healthy for their age.

The cell lines do not release high levels of reverse transcriptase into the medium.

### Characterization of the Epo-dependent Erythroid Cell Lines

The three Epo-dependent cell lines have been passed for >1 yr in cultures supplemented with Epo alone. In these cultures, benzidine-positive cells were routinely detected, although their frequency varied between the cell lines (Table III). In the presence of Epo, the cells also formed colonies containing benzidine-positive cells in semisolid medium (Table I).

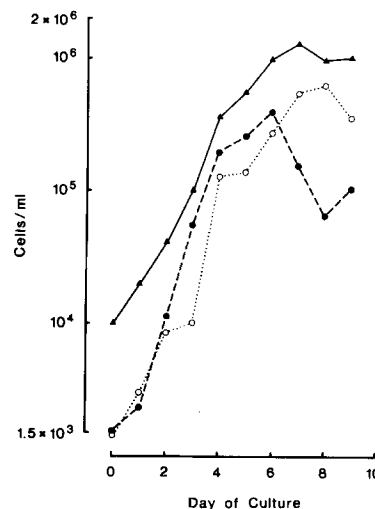
To better understand how a cell line could differentiate and still be passed in culture in the presence of Epo, we determined the number of cells which gave rise to colonies in semisolid medium, the percent of benzidine-positive cells, and the total number of cells per flask during passage in liq-

uid culture. A representative experiment using 32D Epo1 cells is shown in Fig. 2. This cell line has a saturation density of  $10^6$  cells/ml and a mean doubling time in log growth phase of 15 h. The percent of cells able to form colonies goes from a maximum of 50% in the log phase of the culture to a minimum of 10% when the cells have reached saturation density. At the same time, the frequency of benzidine positive cells goes from a minimum of 10-15% at the beginning of the passage to 50-60% at the end of the culture. These results indicate that at the beginning of the culture the population has a higher proportion of cells with high proliferative potential and which form colonies in semisolid cultures while at saturation density a greater percentage of the cell population is differentiated.

The Epo-responsive cell lines grow equally well in FBS-deprived cultures. However, they differed in their capacity to form colonies in response to Epo or other hematopoietic growth factors (Table I), in their capacity to grow in FBS-supplemented cultures (Table I and Fig. 3), and in the maximal frequency of differentiated cells observed at the end of the passage (Table III).

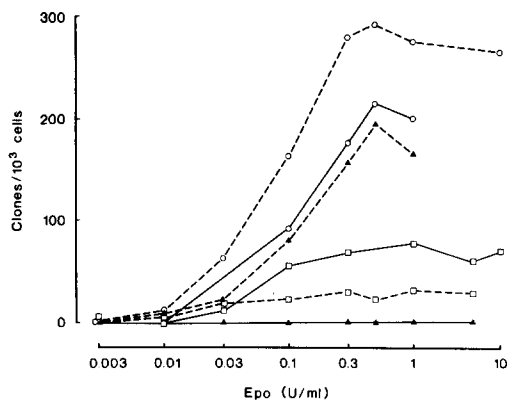
The cloning efficiency in FBS-deprived cultures supplemented with Epo ranged from 1% for the 32D Epo3 to 30% for the 32D Epo1 (Table I and Fig. 3). Maximal cloning efficiency was observed between 0.3 and 1.0 U of Epo/ml for all the cell lines.

One of the cell lines, 32D Epo2, cloned equally well in the presence of Epo or IL-3 and also formed colonies in the presence of GM-CSF or G-CSF, but only at a low frequency and only under FBS-deprived conditions (Table I). The other two cell lines, 32D Epo1 and 3, did not respond to GM-CSF or G-CSF and formed colonies in the presence of IL-3 with a cloning efficiency that was one-tenth to one-third that of 32D cl 3 or to their cloning efficiency in Epo-supplemented cultures (Table I and Fig. 4). The cells responding to IL-3 or Epo may represent the same subset of cells because the number of colonies observed was the same in cultures sup-



**Figure 2.** Growth curves of the 32D Epo1 cell line in FBS-deprived culture supplemented with Epo (1 U/ml). The total number of cells ( $\blacktriangle$ ), the number of cells which give rise to colonies in semisolid medium ( $\bullet$ ), and the number of benzidine-positive cells ( $\circ$ ) per milliliter of culture are shown. The data points represent the mean of two separate experiments performed in duplicate.





**Figure 3.** The effect of increasing concentrations of Epo on the cloning efficiency of 32D Epo1 (□), 32D Epo2 (○), and 32D Epo3 (▲) in FBS-supplemented (—) and FBS-deprived (---) cultures. The data points represent the mean of two separate experiments performed in duplicate.

plemented with Epo, IL-3, or with both (data not shown). The colonies formed in the presence of IL-3 alone were always benzidine negative (data not shown).

The effect of the presence of FBS was again variable. 32D Epo1 formed more colonies in the presence of FBS while 32D Epo2 formed slightly more colonies in FBS-deprived cultures. 32D Epo3 failed to grow in the presence of FBS (Fig. 3).

The heterogeneity in the capacity of the cells to differentiate in response to Epo was reflected also by the erythroid markers detected by Northern analysis. In the presence of Epo, 32D Epo1 contained detectable mRNAs for  $\beta$ -globin and carbonic anhydrase I while 32D Epo2 contained detectable message for carbonic anhydrase I only (Fig. 5). Neither cell line contained mRNA for carbonic anhydrase II (data not presented). The original 32D cl 3, as well as 32D cl 3(G), failed to express detectable transcripts for these two genes (Fig. 5).

The erythroid lines were negative for the myeloid markers investigated (Table III).

### Characterization of the GM-CSF- and G-CSF-dependent Cell Lines

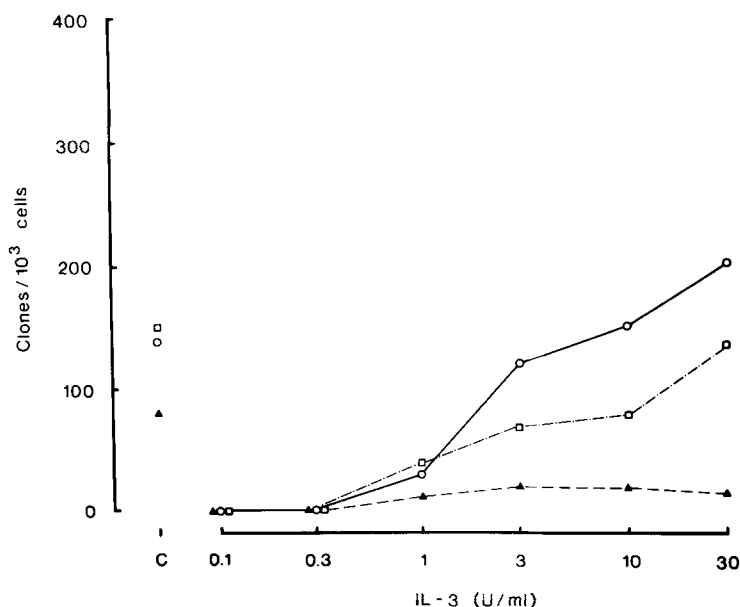
The myeloid cell lines have been passed for >1 yr in FBS-deprived cultures supplemented only with GM-CSF or G-CSF. Their growth properties (saturation density; mean doubling times) were similar to those shown in Fig. 2 for the Epo-dependent cell lines.

In the presence of GM-CSF, the GM-CSF-dependent cell lines had a cloning efficiency of ~20% (Table I and Fig. 6 a). They responded equally well to IL-3 but did not grow in G-CSF (Table I and Fig. 4). Their morphology (Fig. 1 c) is generally that of myeloblasts which express nonspecific esterase at high frequency (13%) and myeloperoxidase and chloroacetate esterase at low frequency (1–2%) (Table III).

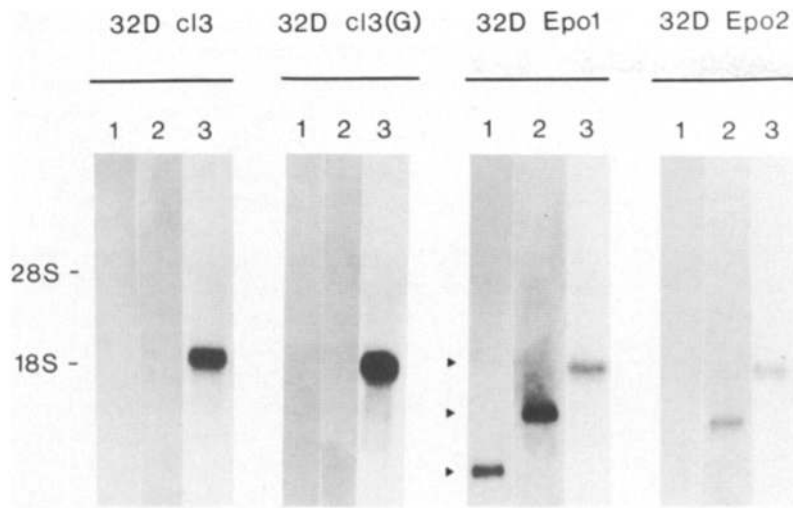
The G-CSF-dependent cell lines grew only in cultures deprived of FBS (Table I and Fig. 6 b). The factor present in the FBS that inhibits their growth has not been identified. In the presence of G-CSF, the G-CSF-dependent cell lines had a cloning efficiency of ~20% (Table I). They did not grow in cultures supplemented with GM-CSF and died after a few divisions in cultures supplemented with IL-3 (Table I). Their morphology (Fig. 1 d) is predominantly that of myelocytes and neutrophilic metamyelocytes which express nonspecific esterase, myeloperoxidase, and, at high frequency (29–44%), chloroacetate esterase (Table III).

### Discussion

Growth factor-dependent cell lines represent a useful tool to study the biochemistry of the intracellular pathway of signal transduction. Although at least several different growth factors mediate proliferation and differentiation of hematopoietic cells (Metcalf, 1986; Clark and Kamen, 1987), most of the nontransformed hematopoietic cell lines described are IL-3 dependent. Epo-dependent (Branch et al., 1987; Saka-



**Figure 4.** The effect of increasing concentrations of IL-3 on the cloning efficiency of 32D cl 3 (○), 32D GM1 (□), and 32D Epo1 (▲) in FBS-deprived cultures. Controls (C) indicate the number of colonies induced under the same culture conditions by Epo, GM-CSF, or PHA-LBRM 33 CM on 32D Epo1, 32D GM1, and 32D cl 3, respectively. The data points represent the mean of two separate experiments performed in duplicate.



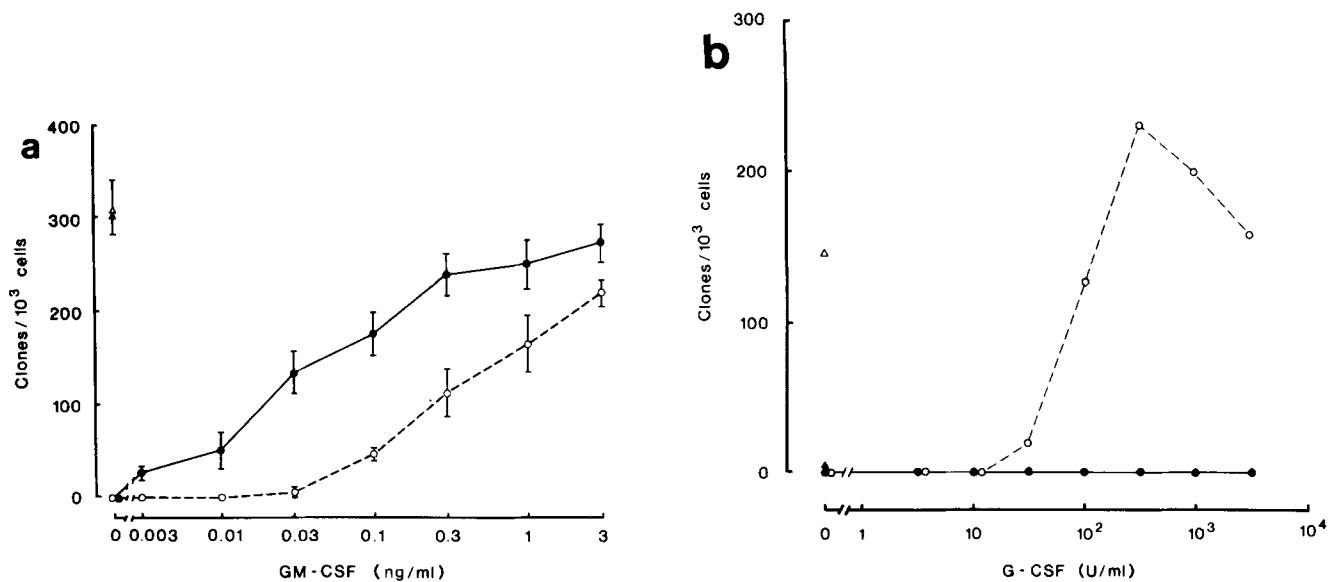
**Figure 5.** Northern blot analysis of total RNA from the original 32D cl 3 and 32D cl 3(G) cell lines (10  $\mu$ g/lane) or from two Epo-dependent cell lines (32D Epo1 and 32D Epo2; 10  $\mu$ g/lane). The blots have been probed for murine  $\beta$ -globin (lanes 1), murine carbonic anhydrase I (lanes 2), or  $\beta$ -actin (lanes 3). The messages are of the sizes expected for  $\beta$ -actin, carbonic anhydrase I, and  $\beta$ -globin, and are indicated by the arrows. The positions of 28S and 18S RNA are also indicated as markers of molecular weight.

guchi et al., 1987) and GM-CSF- or G-CSF-dependent (Weinstein et al., 1986) cell lines have been reported. However, these cell lines are tumorigenic and differentiate poorly, if at all, in the presence of physiologic stimuli.

Cell responsiveness to growth factors may be assessed by several criteria; these are (in order of increasing stringency): incorporation of tritiated thymidine, increment in the number of cells after short term incubation in suspension culture, and induction of colony formation in semisolid cultures or maintenance of the cell line, over a period of months, in suspension culture. These criteria are related to the capacity of a growth factor to initiate, in order, DNA synthesis, cell progression along the cell cycle, proliferation events which result in cells capable of at least limited proliferation, and

proliferation events which result in self-renewal. A further criterion of cell responsiveness to a growth factor is the induction of specific gene expression. It is not clear if these different effects are mediated through the activation of at least partially different intracellular pathways or are dependent on the intrinsic program of the target cell.

It is well-known that IL-3-dependent cells may be conditioned to respond to other growth factors. In fact, IL-3-dependent cell lines cultured for 1-2 mo in the presence of IL-3 plus another growth factor, such as IL-2 (Le Gros et al., 1985), G-CSF (Rovera et al., 1989), or Epo (Sakaguchi et al., 1987; Branch et al., 1987) are conditioned to respond to the second growth factor. The extent of the response is not homogeneous. For example, IL-2 maintains indefinitely IL-



**Figure 6.** (a) The effect of increasing concentrations of GM-CSF on the cloning efficiency of 32D GM1 in FBS-supplemented (●) and FBS-deprived (○) cultures. Similar results were obtained with 32D GM2 cells. The number of colonies observed in FBS-supplemented (▲) and FBS-deprived (△) cultures stimulated with PHA-LBRM CM are shown for comparison. The data points represent the mean of two separate experiments performed in duplicate. (b) The effect of increasing concentrations of G-CSF on the cloning efficiency of 32D G1 in FBS-supplemented (●) and FBS-deprived (○) cultures. Similar results were obtained with 32D G2 cells. The number of colonies observed in FBS-supplemented (▲) and FBS-deprived (△) cultures stimulated with IL-3 are shown for comparison. The data points represent the mean of two separate experiments performed in duplicate.

2-conditioned 32D cl 23 cells in suspension culture but the morphology of these cells is unchanged from the morphology of the same cells growing in IL-3. G-CSF induces neutrophil differentiation of G-CSF-conditioned 32D cl 3 cells but is unable to sustain their proliferation for more than four to five divisions. Epo induces proliferation of Epo-conditioned DA-1 cells but has no effect on their differentiation. Another example is the FDCP-1 cell line which, when established, did not grow in GM-CSF (Dexter et al., 1980) and may have acquired this capacity during passage in WEHI-3 conditioned medium which contains both IL-3 and GM-CSF. FDCP-1 does not differentiate in GM-CSF, however.

The cell lines presented here are the first immortalized lines which grow indefinitely in FBS-deprived cultures supplemented with Epo, GM-CSF, or G-CSF and which partially differentiate along the appropriate lineages in the presence of these growth factors. Furthermore, we have not found significant differences between the concentrations of the growth factors which induce plateau colony growth of these cell lines and the corresponding concentrations required for plateau colony growth of normal murine progenitor cells under the same culture conditions (Migliaccio et al., 1988, 1989). The features of the Epo-, GM-CSF-, and G-CSF-dependent cell lines are consistent with what is known of the biological activity of the growth factors on murine cells. The Epo-dependent cell lines acquire the morphology of erythroblasts, are positive for carbonic anhydrase I and/or  $\beta$ -globin gene expression, and are negative for myeloperoxidase and chloroacetate esterase. The GM-CSF- and G-CSF-dependent cell lines have predominantly the morphology of myeloblasts or metamyelocytes, respectively. They are positive for myeloperoxidase and chloroacetate esterase and negative for  $\beta$ -globin and carbonic anhydrase gene expression. Interestingly, a fraction of the GM-CSF-dependent cells resembles monoblasts. We have not analyzed these cells for the expression of markers specific for this lineage. These data suggest that the Epo-dependent erythroid or GM-CSF- and G-CSF-dependent myeloid cell lines represent two subpopulations of cells committed toward erythroid or myeloid differentiation, respectively. Consistent with this is the fact that, in general, the erythroid cell lines do not clone in the presence of GM-CSF or G-CSF and the myeloid cell lines do not clone in the presence of Epo. Furthermore, it has not been possible to date to isolate GM-CSF- or G-CSF-dependent cell lines from the Epo-dependent line or vice versa.

The three erythroid cell lines are heterogeneous with respect to sensitivity to other hematopoietic growth factors and the degree of differentiation observed in culture. 32D Epo2 grows equally well in Epo and IL-3, and responds to some extent to GM-CSF and G-CSF. At the beginning of the passage, these cells do not express levels of  $\beta$ -globin message detectable by Northern analysis but express carbonic anhydrase I, a gene activated at an early stage of erythroid differentiation. At the end of the passage, only 5–10% of the 32D Epo2 cells are benzidine positive. In contrast, 32D Epol has a lower cloning efficiency in IL-3 than in Epo, does not respond to GM-CSF or G-CSF, expresses detectable levels not only of carbonic anhydrase I but also of  $\beta$ -globin message, and >50% of the cells are benzidine-positive at the end of passage. These results suggest that 32D Epol is more differentiated than 32D Epo2.

The mechanism underlying the conditioning of IL-3-dependent cell lines to respond to other growth factors may be represented either by induction of a high affinity type of receptor, as is the case for IL-2 (De Gros et al., 1987), or by increasing an otherwise low number of high affinity receptors, as is the case for Epo (Sakaguchi et al., 1987). On the parental line 32D cl 3, Epo receptors are almost undetectable. In contrast, ~400 high affinity Epo receptors are detectable on the 32D Epol cell line (Migliaccio et al., 1989). This number of receptors is comparable to the number present on the normal rodent erythroid colony-forming unit (Mayeux et al., 1987; Mufson and Gesner, 1987).

For most of the IL-3-dependent cell lines described, nothing is known of the molecular events which led to cell immortalization. One exception is the NFS-60 cell line (Weinstein et al., 1986), which was obtained from mice infected with the CasBrM virus. This cell line expresses a truncated version of the myb protein because the long terminal repeat sequences of the virus are inserted into the *c-myb* locus. Regardless of the molecular event which led to immortalization of the IL-3-dependent cell lines, the stochastic model of hematopoietic differentiation (Korn et al., 1973) predicts that the result is an increased frequency of self-replicative cell divisions. Differentiative cell divisions, however, would still be possible, and cells responding to hematopoietic growth factors other than IL-3 would be continuously generated in culture, although at a low frequency. This prediction was verified in our experiments. Epo-, GM-CSF-, and G-CSF-dependent cell lines were regularly isolated from 32D cl 3 and cl 3(G). The frequency of the cells which give rise to these cell lines in the original cell lines is very low, approximately one in  $10^5$  cells. This frequency is lower than the frequency of cells which give rise to colonies in response to Epo, GM-CSF, or G-CSF in the same cell populations and which correspond to approximately one in  $10^2$ – $10^4$  cells. This suggests that only a fraction of cells which proliferate in response to Epo, GM-CSF, or G-CSF have the capacity to self-replicate in response to these growth factors. Our results also indicate that the original immortalization event is inherited in a dominant fashion by 32D cells, although by a minority of them, independent of the stimulus to which they became programmed to respond. For these reasons, it is unlikely that such an event involves the cellular postreceptor pathway.

In conclusion, the immortalized cell lines described here may be classified at different stages of hematopoietic differentiation on the basis of the growth factors to which they respond, the level and the type of differentiation that they achieve, and the possibility to generate cells which respond to factor(s) different from that in which they normally grow. Since these cell lines all have the same genotype, but apparently are "committed" to different differentiation programs, they may prove useful in studies of the biochemical and molecular events underlying the process of hematopoietic differentiation.

We would like to acknowledge Dr. J. J. Mermod and Dr. L. Souza for providing us with pure recombinant growth factors. Dr. J. Pierce is acknowledged for providing data of the karyotype of the 32D cl 3 cell line. Dr. P. Curtis is acknowledged for providing us the cDNA for murine  $\beta$ -globin and carbonic anhydrase I and II.

This study was supported by research grants CA-31615 and 2POICA-21124-11 from the National Institutes of Health, Department of Health and



Human Services. G. Migliaccio and A. R. Migliaccio have been supported by the Associazione Italiana Ricerca sul Cancro and by the European Organization for Research Training on Cancer, respectively, during a leave of absence from the Istituto Superiore di Sanità, Rome, Italy. J. W. Adamson is an American Cancer Society Clinical Research Professor.

Received for publication 3 January 1989 and in revised form 12 April 1989.

## References

- Branch, D. R., J.-M. Turc, and L. J. Guilbert. 1987. Identification of an erythropoietin-sensitive cell line. *Blood*. 69:1782-1785.
- Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry*. 18:5294-5299.
- Clark, S. C., and R. Kamen. 1987. The human hematopoietic colony-stimulating factors. *Science (Wash. DC)*. 236:1229-1236.
- Curtis, P. J. 1983. Cloning of mouse carbonic anhydrase mRNA and its induction in mouse erythroleukemic cells. *J. Biol. Chem.* 258:4459-4463.
- Dexter, T. M., J. Garland, D. Scott, E. Scolnick, and D. Metcalf. 1980. Growth of factor-dependent hemopoietic precursor cell lines. *J. Exp. Med.* 152:1036-1047.
- Egrie, J. C., J. K. Browne, P. Lai, and F.-K. Lin. 1985. Characterization of recombinant monkey and human erythropoietin. In *Experimental Approaches for the Study of Hemoglobin Switching*. G. Stamatoyannopoulos and A. W. Nienhuis, editors. Alan R. Liss, Inc., New York. 339-350.
- Fraser, P. J., and P. J. Curtis. 1986. Molecular evolution of the carbonic anhydrase genes: calculation of divergence time for mouse carbonic anhydrase I and II. *J. Mol. Evol.* 23:294-299.
- Gillis, S., M. Scheid, and J. Watson. 1980. Biochemical and biologic characterization of lymphocyte regulatory molecules. III. The isolation and phenotypic characterization of interleukin-2 producing T cell lymphomas. *J. Immunol.* 125:2570-2578.
- Greenberger, J. S., P. Gans, P. Davisson, and W. Moloney. 1979. In vitro induction of continuous acute promyelocytic leukemia cell lines by Friend or Abelson murine leukemia virus. *Blood*. 53:987-1001.
- Greenberger, J. S., R. Eckner, W. Ostertag, B. Colletta, S. Boshetti, H. Nagasawa, A. Karpas, R. Weichselbaum, and W. Moloney. 1980a. Release of spleen focus-forming virus (SFFV) from differentiation inducible promyelocytic leukemia cell lines transformed in vitro by Friend leukemia virus. *Virology*. 105:425-435.
- Greenberger, J. S., P. Newburger, J. Lipton, M. Sakakeeny, and W. Moloney. 1980b. Leukemogenesis in long-term bone marrow cultures of NIH Swiss [N:NIH(S)] mice. *Natl. Cancer Inst.* 64:867-878.
- Greenberger, J. S., M. A. Sakakeeny, R. K. Humphries, C. J. Eaves, and R. J. Eckner. 1983. Demonstration of permanent factor-dependent multipotential (erythroid/neutrophil/basophil) hematopoietic progenitor cell lines. *Proc. Natl. Acad. Sci. USA*. 80:2931-2935.
- Gualtieri, R. J., C. M. Liang, R. K. Shaddock, A. Waheed, and J. T. I. Banks. 1987. Identification of the hematopoietic growth factors elaborated by bone marrow stromal cells using antibody neutralization analysis. *Exp. Hematol.* 15:883-889.
- Kelso, A. 1986. An assay for colony-stimulating factor (CSF) production by single T lymphocytes: estimation of the frequency of cells producing granulocyte-macrophage CSF and multi-lineage CSF within a T lymphocyte clone. *J. Immunol.* 136:2930-2937.
- Korn, A. P., R. M. Henkelman, R. P. Ottensmeyer, and J. E. Till. 1973. Investigations of a stochastic model of hemopoiesis. *Exp. Hematol.* 1:362-375.
- Koyasu, S., J. Yodoi, T. Nikaïdo, Y. Tagaya, Y. Taniguchi, T. Honjo, and I. Yahara. 1986. Expression of interleukin 2 receptors on interleukin 3-dependent cell lines. *J. Immunol.* 136:984-987.
- Le Gros, G. S., S. Gillis, and J. D. Watson. 1985. Induction of IL 2 responsiveness in a murine IL 3-dependent cell line. *J. Immunol.* 135:4009-4014.
- Le Gros, G. S., P. S. Shackell, J. E. Le Gros, and J. D. Watson. 1987. Interleukin 2 regulates the expression of IL-2 receptors on interleukin 3-dependent bone marrow-derived cell lines. *J. Immunol.* 138:478-483.
- Lowenthal, J. W., B. E. Castle, J. Christiansen, J. Schreurs, D. Rennick, N. Arai, P. Hoy, Y. Takebe, and M. Howard. 1988. Expression of high affinity receptors for murine interleukin 4 (BSF-1) on hemopoietic and non-hemopoietic cells. *J. Immunol.* 140:456-464.
- Mayeux, P., C. Billat, and R. Jacquot. 1987. The erythropoietin receptor of rat erythroid progenitor cells: characterization and affinity cross-linkage. *J. Biol. Chem.* 262:13985-13990.
- Metcalf, B. 1985. Multi-CSF-dependent colony formation by cells of a murine hemopoietic cell line: specificity and action of multi-CSF. *Blood*. 65:357-362.
- Metcalf, D. 1986. The molecular biology and functions of the granulocyte-macrophage colony-stimulating factors. *Blood*. 67:257-267.
- Metcalf, D., A. W. Burgess, G. R. Johnson, N. A. Nicola, E. C. Nice, J. De Lamarer, D. R. Thatcher, and J.-J. Mermod. 1986a. In vitro actions on hemopoietic cells of recombinant murine GM-CSF purified after production in *Escherichia coli*: comparison with purified native GM-CSF. *J. Cell Physiol.* 128:421-431.
- Metcalf, D., C. G. Begley, G. R. Johnson, N. A. Nicola, A. F. Lopez, and D. J. Williamson. 1986b. Effects of purified bacterially synthesized murine multi-CSF (IL-3) on hematopoiesis in normal adult mice. *Blood*. 68:46-57.
- Migliaccio, G., and A. R. Migliaccio. 1987a. Cloning of human erythroid progenitors (BFU-E) in the absence of fetal bovine serum. *Br. J. Hematol.* 67:129-133.
- Migliaccio, G., and A. R. Migliaccio. 1987b. Early hemopoietic differentiation: the action of multi-CSF is complemented by lineage specific growth factors. *Ann. NY Acad. Sci.* 511:39-49.
- Migliaccio, G., A. R. Migliaccio, and J. W. M. Visser. 1988. Synergism between erythropoietin and interleukin-3 in the induction of hemopoietic stem cells proliferation and erythroid burst formation. *Blood*. 42:944-951.
- Migliaccio, G., A. R. Migliaccio, K. Kaushansky, and J. W. Adamson. 1989. Regulation of differentiation of murine progenitor cells derived from blast cell colonies under serum-deprived conditions. *Exp. Hematol.* 17:110-115.
- Migliaccio, G., A. R. Migliaccio, V. Broudy, B. L. Krieger, G. Rovera, and J. W. Adamson. 1989. Selection of lineage-restricted cell lines immortalized at different stages of hematopoietic differentiation from the murine cell line 32D. In *Proceedings of the Sixth Conference of Hemoglobin Switching*. G. Stamatoyannopoulos and A. W. Nienhuis, editors. Alan R. Liss, Inc., New York. In press.
- Mufson, R. A., and T. G. Gesner. 1987. Binding and internalization of recombinant human erythropoietin in murine erythroid precursor cells. *Blood*. 69:1485-1490.
- Naparstek, B., J. Pierce, D. Metcalf, R. Shaddock, J. Ihle, A. Leder, M. A. Sakakeeny, K. Wagner, J. Falco, T. J. FizGerald, and J. S. Greenberger. 1986. Induction of growth alterations in factor-dependent hematopoietic progenitor cell lines by cocultivation with irradiated bone marrow stromal cell lines. *Blood*. 67:1395-1403.
- Nudel, U., J. Salmon, E. Fibach, M. Terada, R. Rifkin, P. A. Marks, and A. Bank. 1977. Accumulation of  $\alpha$ - and  $\beta$ -globin messenger RNAs in mouse erythroleukemia cells. *Cell*. 12:463-469.
- Ohara, J., and W. E. Paul. 1987. Receptors for B-cell stimulatory factor-1 expressed on cells of haematopoietic lineage. *Nature (Lond.)*. 325:537-540.
- Orkin, S. H., F. I. Harosi, and P. Leder. 1975. Differentiation in erythroleukemic cells and their somatic hybrids. *Proc. Natl. Acad. Sci. USA*. 72:98-102.
- Park, L. S., D. Friend, K. Grabstein, and D. L. Urdal. 1987. Characterization of the high-affinity cell-surface receptor for murine B-cell-stimulating factor 1. *Proc. Natl. Acad. Sci. USA*. 84:1669-1673.
- Rovera, G., B. Kreider, N. Shirsat, D. Venturelli, G. Naso, and F. Mavilio. 1989. Alteration of the program of terminal differentiation caused by oncogenes in the hemopoietic progenitor cell line 32D C13 (G). *Ann. NY Acad. Sci.* In press.
- Sakaguchi, M., Y. Koishihara, H. Tsuda, K. Fujimoto, K. Shibuya, M. Kawakita, and K. Takatsuki. 1987. The expression of functional erythropoietin receptors on an interleukin-3 dependent cell line. *Biochem. Biophys. Res. Commun.* 146:7-12.
- Souza, L. M., T. C. Boone, J. Gabilove, P. H. Lai, K. M. Zsebo, D. C. Murdoch, V. R. Chazin, J. Bruszewski, H. Lu, K. K. Chen, J. Barendt, E. Platzer, M. A. S. Moore, R. Mertelmann, and K. Welte. 1986. Recombinant human granulocyte colony-stimulating factor: the effect on normal and leukemic myeloid cells. *Science (Wash. DC)*. 232:61-65.
- Thomas, P. S. 1980. Hybridization of denatured RNA and small DNA fragments transferred to ribocellulose. *Proc. Natl. Acad. Sci. USA*. 77:5201-5205.
- Tsao, C.-J., A. Tojo, H. Fukamachi, T. Kitamura, T. Saito, A. Urabe, and F. Takaku. 1988. Expression of the functional erythropoietin receptors on interleukin 3-dependent murine cell lines. *J. Immunol.* 140:89-93.
- Valtieri, M., D. J. Tweardy, D. Caracciolo, K. Johnson, F. Mavilio, S. Altman, D. Santoli, and G. Rovera. 1987. Cytokine-dependent granulocytic differentiation: regulation of proliferative and differentiative responses in a murine progenitor cell line. *J. Immunol.* 138:3829-3835.
- Watson, J. D., P. S. Crosier, C. J. March, P. J. Conlon, D. Y. Mochizuki, S. Gillis, and D. L. Urdal. 1986. Purification to homogeneity of a human hematopoietic growth factor that stimulates the growth of a murine interleukin 3-dependent cell line. *J. Immunol.* 137:854-857.
- Weinstein, Y., J. N. Ihle, S. Lavu, and E. P. Reddy. 1986. Truncation of the c-myc gene by a retroviral integration in an interleukin 3-dependent myeloid leukemia cell line. *Proc. Natl. Acad. Sci. USA*. 83:5010-5014.