ACTIVATION AND GROWTH OF COLONY-STIMULATING FACTOR–DEPENDENT CELL LINES IS CELL CYCLE STAGE DEPENDENT

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Colony-stimulating factors (CSF)[1] are a family of glycoproteins that support the clonal proliferation and differentiation of hematopoietic progenitor cells in vitro (1–3). As pluripotent stem cells generate committed progenitor cells of a specific lineage, the number of soluble factors capable of inducing proliferation and/or differentiation of these cells becomes more restricted. This observation has allowed CSFs to be broadly classified into either multilineage or lineage-specific growth factors. Lineage-specific CSFs are those acting on cells capable of forming only one differentiated cell type (4). Granulocyte-CSF (G-CSF), macrophage-CSF (M-CSF), or erythropoietin (EPO) are lineage-specific CSFs that stimulate the proliferation and differentiation of committed progenitor cells into granulocytes, macrophages, or erythrocytes, respectively (5–7). Multilineage CSFs are those stimulating primitive multipotent cells that generate more than one fully differentiated cell type. Granulocyte/macrophage-CSF (G/M-CSF) and IL-3 (multi-CSF) are two distinct multilineage CSFs that support the proliferation of a broad distribution of hematopoietic cell types. G/M-CSF can stimulate the production of mature granulocyte, macrophage, or mixed colonies from bone marrow progenitor cells (8, 9). It also initiates, but does not sustain, proliferation of some multipotential erythroid and megakaryocyte precursors (10). IL-3 induces colony formation of multiple cell types in cultures of bone marrow or fetal liver cells. It supports the growth and/or differentiation of multipotent precursor cells belonging to both the myeloid and lymphoid lineages as well as more differentiated progenitor cells belonging to the myeloid lineage (2, 11–15). IL-3 has also been associated with multiple activities and has been termed burst-promoting activity (16), mast cell growth factor (17, 18), P cell stimulating factor (19, 20), and histamine-producing cell-stimulating factor (21) by various investigators.

The broad spectrum of activities associated with IL-3 suggests that it may play a role in both the self-renewal of multipotent stem cells as well as the growth and differentiation of committed progeny. However, the mechanism by which IL-3 mediates these processes is not understood. In this report, we have studied the

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[1] *Abbreviations used in this paper:* BSF-1, B cell stimulatory factor 1; CM, conditioned medium; CSF, colony-stimulating factor; EPO, erythropoietin; G-CSF, granulocyte-CSF; G/M-CSF, granulocyte/macrophage CSF; IMDM, Iscove's modified Dulbecco's medium; M-CSF, macrophage-CSF.

growth and differentiation of two IL-3-dependent cell lines, FDC-P1 (a myeloid progenitor) (22) and FL5.12 (a lymphoid progenitor) (14). FDC-P1 was generated from normal bone marrow cells cultured in the presence of WEHI-3 conditioned medium (CM) and appears to represent a cell early in myelomonocytic differentiation (22). FL5.12 was generated from the culture of 13-d fetal liver cells in the presence of WEHI-3 CM and represents a multipotential B lymphoid precursor cell since these cells can be induced to generate mature IgM-secreting B cells committed to several different variable region specificities (14). Both these clones are nonmalignant since they do not form tumors in nude mice. These clones can be rendered factor-independent and tumorigenic by retrovirus infection (23). The response to IL-3 and other hematopoietic CSFs was investigated among activated and resting cells. A model for the regulation of cell growth and differentiation by IL-3 as well as other CSFs is presented.

Materials and Methods

Cell Lines. The IL-3-dependent cell lines, FDC-P1 and FL5.12, were maintained in Iscove's modified Dulbecco's medium (IMDM; E. I. du Pont de Nemours & Co.) supplemented with 10% FCS (HyClone Laboratories, Logan, UT) and 10% WEHI-3B(D⁻) CM as a source of IL-3. Cell lines were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air.

Growth Factors. WEHI-3B(D⁻) supernatant, which contains IL-3 (24), was stored at −20°C following centrifugation at 500 g for 15 min and passage through a 0.22-μm filter. This supernatant, added at 10% (vol/vol) to IMDM containing 10% FCS, will be referred to as WEHI-3 CM. Recombinant G/M-CSF (mouse) (rG/M-CSF), sp act 5×10^7 CFUs/mg protein, and purified mouse IL-3 supplied at 5,000 U/ml were obtained from Genzyme (Boston, MA). One CFU is defined as that amount of growth factor that produces a single colony from 7.5×10^4 murine bone marrow cells in semisolid medium. These units correlated with our titration in proliferation assays where 1 U is the amount of factor required to induce 50% maximal [³H]TdR uptake. Purified human rIL-2 and purified human rIL-1β were obtained from E. I. DuPont Co. (Wilmington, DE). Recombinant B cell stimulatory factor 1 (BSF-1, IL-4) was kindly provided by Dr. Donna Rennick (DNAX, Palo Alto, CA); and purified natural BSF-1 (IL-4) was kindly provided by Drs. Neal Roehm and John Cambier (National Jewish Hospital, Denver, CO).

Cell Preparations. FDC-P1 and FL5.12 cells, maintained in WEHI-3 CM or other CSFs, were harvested during log phase of growth, washed four times in HBSS (pH 7.2), and replated at 2×10^5 cells/ml in CSF-supplemented or CSF-deficient medium. After a 24-h incubation at 37°C in 5% CO_2 in air, the cells were harvested, washed four times in HBSS, and live cells (determined by method described below) were collected by Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) density gradient centrifugation. Briefly, 2 $\times 10^8$ washed cells were resuspended in 3 ml of pH 7.4 isoosmotic Percoll ($p = 1.075$). 1 ml HBSS was layered on top and tubes were centrifuged at room temperature for 30 min at 1,400 g. Cells with $p < 1.075$ formed an interface layer between the Percoll and HBSS and all were shown to be dead by trypan blue dye exclusion. Live cells (typically >98%), as determined by trypan blue dye exclusion, were harvested from the pelleted fraction ($p > 1.075$). This preparation of cells was subsequently used in all the individual assays described.

[³H]TdR Uptake. Cell division was measured by resuspending 10^4 washed, viable cells in 0.1-ml cultures in 96-well flat-bottomed microtiter plates (Costar, Cambridge, MA). The plates were then incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. After the indicated time, triplicate cultures were pulsed with 0.5 μCi (sp act 6.7 Ci/mmol; New England Nuclear, Boston, MA) of [³H]TdR, and harvested 4 h later on glass fiber filters with an automatic cell harvester (Cambridge Technology Inc., Cambridge, MA). Results are expressed as mean cpm (± SEM) of [³H]TdR uptake per culture in triplicate
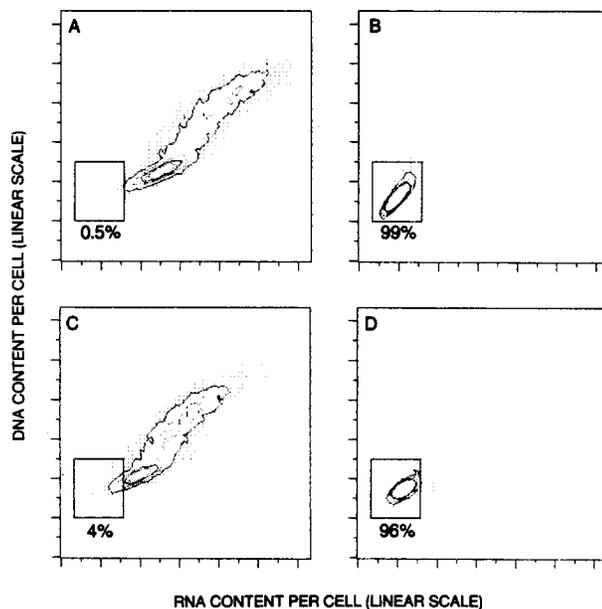
FIGURE 1. Cell cycle compartments in actively cycling or factor-deprived cell lines. FDC-P1 (*A* and *B*) or FL5.12 (*C* and *D*) cells, either actively cycling (*A* and *C*) or 24-h factor-deprived (*B* and *D*) were analyzed for DNA and RNA content using the dye acridine orange. x-axis, red flourescence intensity, RNA content per cell; y-axis, green flourescence intensity, DNA content per cell. Box designates G_0.

groups. In addition, cell number and viability (determined by trypan blue dye exclusion) were monitiored.

Oxidative Phosporylation Enzyme Activity. Oxidative phosphorylation enzyme levels were evaluated as previously described (25). Briefly, cells were prepared and plated in the same manner as for the [³H]TdR uptake assay except that 10^5 cells were plated per well. After the indicated time, 0.02 ml of 3(4,5-dimethyl-thiazoyl-2-yl) 2,5-diphenyltetrazolium bromide (MTT; 5 mg/ml in PBS) (Sigma Chemical Co., St. Louis, MO) was added to each well. Plates were then incubated at 37°C for 4 h. The plates were centrifuged, the supernatant removed, and 0.1 ml of acid-isopropanol (0.04 N HCl in isopropanol) was added and mixed. Plates were read on a Flow Multiscan (Flow Laboratories, Inc., McLean, VA) at a test wavelength of 570 nm and a reference wavelength of 630 nm.

DNA and RNA Content. Simultaneous detection of RNA and DNA levels within single cells was performed using a protocol adapted from Darzynkiewicz et al. (26). Briefly, 10^6 washed, viable cells were resuspended in 0.2 ml HBSS containing 1% FCS. 0.4 ml of 0.08 M HCl, 0.15 M NaCl, and 0.1% Triton X-100 was added, mixed gently, and incubated for 90 s. Acridine orange solution (12 μg/ml) in 0.25 M Na_2HPO_4, 0.1 M citric acid, 0.2 mM EDTA, and 0.15 M NaCl were then added, and incubated at room temperature, protected from light, for 20 min. 37% ethanol was added and the samples were analyzed using a FACS Analyzer (Becton Dickinson & Co., Mountain View, CA) and Consort 30 computer.

Results

Transition to G_0 of IL-3-dependent Cell Lines upon Removal of Ligand. FDC-P1 and FL5.12 cells, maintained in WEHI-3 CM, were harvested, washed in HBSS, and cultured at 2×10^5 cells/ml in the presence or absence of WEHI-3 CM. After 24 h, DNA and RNA content of single cells was quantitated by FACS analysis. During exponential growth phase (Fig. 1, *A* and *C*), ~55, 25, and 20% of these cells are in the G_1, S, and G_2 + M phases of the cell cycle, respectively. However, when these clones were removed from WEHI-3 CM for >20 h (Fig. 1, *B* and *D*), >95% of all cells exit from the cell cycle and accumulate in G_0. Both

FDC-P1 and FL5.12 cells grow in serum-free medium in the presence of WEHI-3 CM. These cells also enter G_0 when deprived of WEHI-3 CM (data not shown).

We confirmed that such cells were in a resting state by monitoring [^{3}H]TdR uptake, oxidative phosphorylation enzyme levels (MTT assay), total cell volume, and number of viable cells. Fig. 2A demonstrates that [^{3}H]TdR uptake begins to decrease 8 h after removal of WEHI-3 CM. Both clones reach background levels of [^{3}H]TdR uptake by 20–24 h of culture. Similarly, oxidative phosphorylation enzyme levels slow dramatically 4–8 h after removal of WEHI-3 CM (Fig. 2B) and negligible levels were observed by 24 h of culture. Total cellular volume measurements determined at 24 h also indicate that a substantial decrease in cell size had occurred (Fig. 2C). Cells in WEHI-3 CM were 15–17 μm in diameter and 7–9 μm in diameter when ligand was removed. 50–70% of the cells were viable 24 h after the removal of WEHI-3 CM (Fig. 2D), and, at 48 h, >50% of FL5.12 cells remain viable, whereas <10% of FDC-P1 cells are viable. No significant increase in cell number was observed when WEHI-3 CM was removed from the culture medium.

Activation of Growth-arrested Clones with WEHI-3 CM. 24-h factor-deprived cells that have accumulated in the G_0 phase of the cell cycle enter cycle when cultured in the presence of WEHI-3 CM. For FDC-P1 cells (Fig. 3A), G_0 to G_1 transition occurs between 8 and 12 h, while the G_1 to S phase transition occurs by 12 h of culture. FL5.12 cells (Fig. 3A), move into G_1 between 4 and 8 h after factor addition, although S phase transition does not occur until ~20 h of culture. Concurrent with the entry of cells into cycle, both [^{3}H]TdR uptake and oxidative phosphorylation enzyme levels increase. FDC-P1 and FL5.12 cells show a significant and steady increase in [^{3}H]TdR uptake beginning at 12–16 h of culture (Fig. 3B), while oxidative phosphorylation enzyme levels increase rapidly after WEHI-3 CM addition (Fig. 3C). Cell viability in these restimulated cultures is maintained at >90% throughout the culture period and a doubling time of ~11–13 h is observed for both cell lines after the initial cell division (Fig. 3D). Both FDC-P1 and FL5.12 cells maintained in the presence of purified IL-3 behave similarly to cells maintained in WEHI-3 CM, suggesting that IL-3 is the factor responsible for cell growth.

[^{3}H]TdR Uptake in IL-3-dependent Clones in Cultures Containing Other CSFs. Several different CSFs were evaluated for their ability to stimulate growth of either activated or resting (G_0) FDC-P1 or FL5.12 cells. FDC-P1 and FL5.12 cells were maintained long-term in either WEHI-3 CM or purified IL-3 and their pattern of responsiveness to other growth factors was similar (Table I). We found that only IL-3 or WEHI-3 CM will support growth of FL5.12 cells (Table I) whether these cells were actively cycling or resting (G_0) cells. These cells do not uptake [^{3}H]TdR in response to human IL-1β, human IL-2, mouse G/M-CSF, or mouse IL-4. In contrast, actively cycling FDC-P1 cells (Table I) respond to WEHI-3 CM or purified IL-3 as well as G/M-CSF or IL-4. Significant increases in [^{3}H]TdR uptake in response to WEHI-3 CM or purified IL-3 can be observed at 8–12 h of culture while G/M-CSF or IL-4 appear to maintain a steady-state level of [^{3}H]TdR uptake over the 24-h culture period (Fig. 4A). These cells do not uptake [^{3}H]TdR in response to either IL-1 or human IL-2 and long-term growth has never been observed under these conditions.
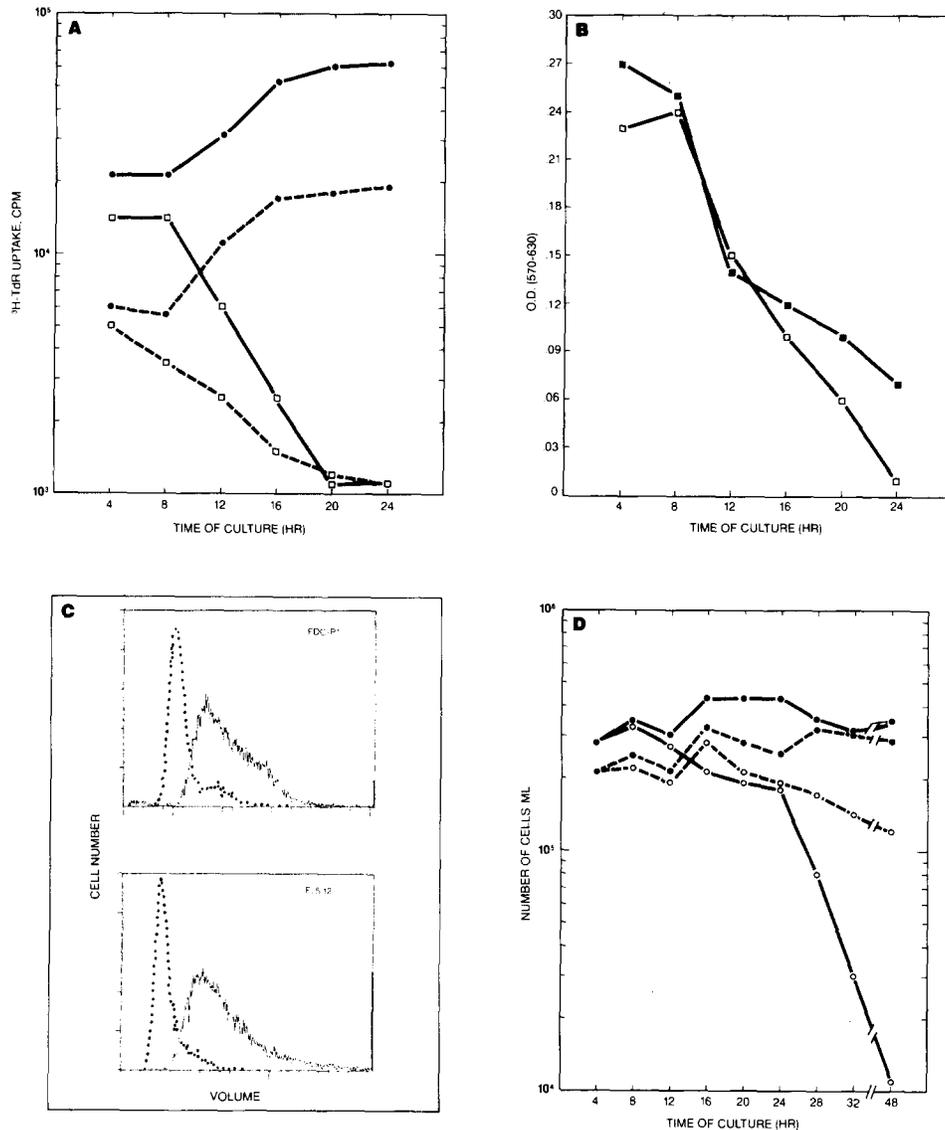
FIGURE 2. Cellular activity in factor-deprived IL-3-dependent cell lines. [³H]TdR uptake (A) in FDC-P1 (———) and FL5.12 (— — —) cells was evaluated over a 24-h culture period in the presence of (□) medium, or (●) WEHI-3 CM. Oxidative phosphorylation enzyme activity (B) was measured over a 24-h culture period in FDC-P1 (■) or FL5.12 (□) cells. Cell volume measurements (C) of actively cycling (———) or 24-h factor-deprived (· · · · ·) cells were analyzed. Cell viability was followed in duplicate cultures (D) by trypan blue dye exclusion in FDC-P1 (———) or FL5.12 (– – –) cells plated in medium (●) total cells, (○) viable cells. Data points represent means of triplicate cultures.

In contrast to activated (G₁→M) FDC-P1 cells, resting (G₀) cells grow only in the presence of WEHI-3 CM or IL-3 (Table I, Fig. 4B). Nearly identical patterns of [³H]TdR uptake, as described above, were obtained from both cell lines, whether actively cycling or resting (G₀), over a 100-fold (2–200 U/ml) range of
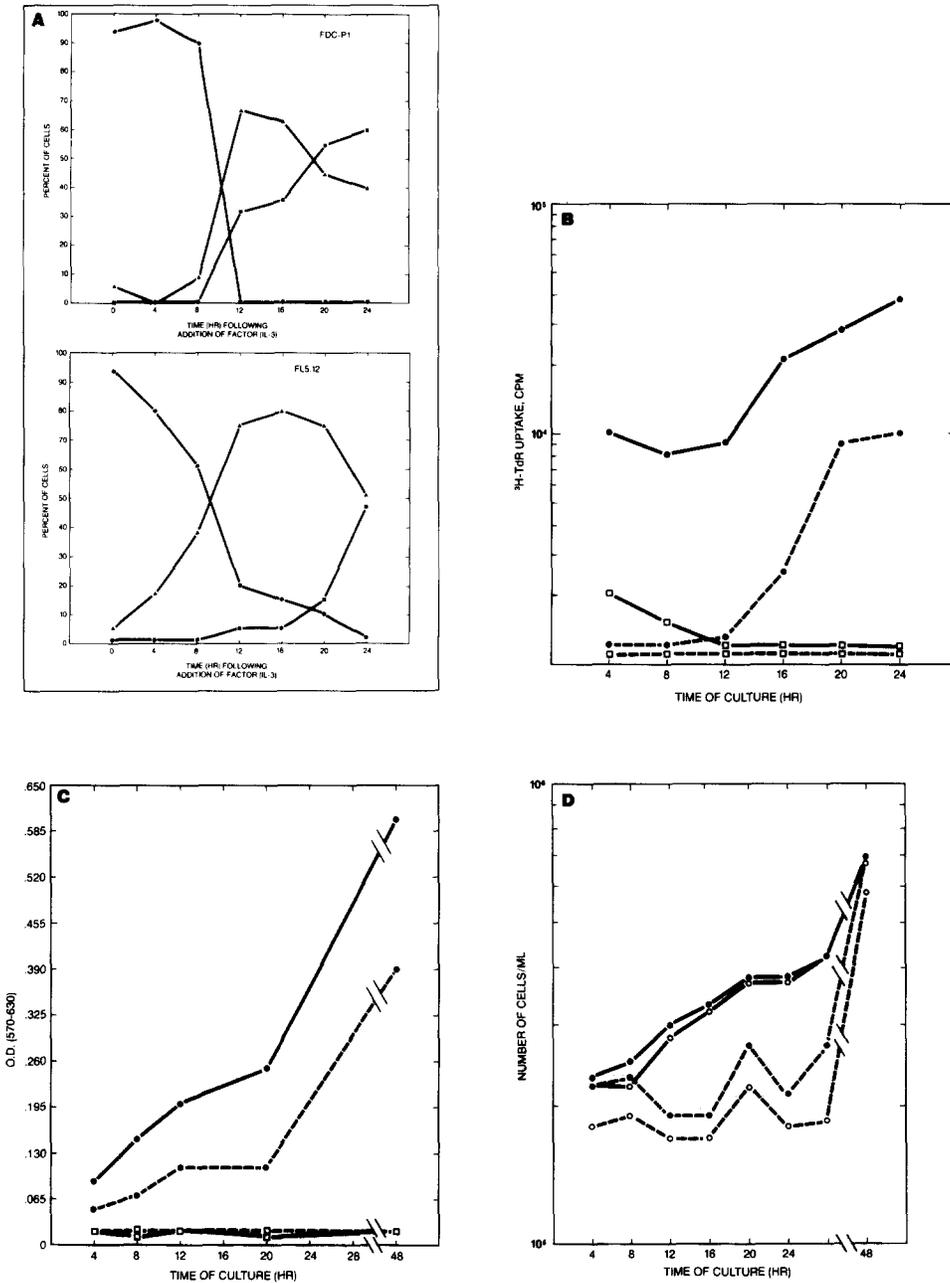
FIGURE 3.   Exit of cells from G_0 after readdition of IL-3. FDC-P1 and FL5.12 cells were factor-deprived for 24 h and replated in the presence of WEHI-3 CM. After the indicated time period, acridine orange staining for analysis of cell cycle compartments was performed (A). (●) G_0, (▲) G_1, (■) S + G_2 + M. The kinetics of induction of [^{5}H]TdR uptake (B) and oxidative phosphorylation enzyme activity (C) in resting (G_0) FDC-P1 (———) or FL5.12 (– – –) cells was followed in the presence of (□) medium, or (●) WEHI-3. Cell viability by trypan blue dye exclusion (D) was similarly followed in FDC-P1 (———) and FL5.12 (– – –) cells. (●) total cells, (○) viable cells. Data points represent mean of triplicate cultures. The SEM was <15%.

TABLE I

[³H]TdR Uptake by IL-3-dependent Cell Lines in Response to Hematopoietic Growth Factors

Cell line*	Factor‡	Culture condition						
		Med§	WEHI-3 CM	IL-3	IL-1	IL-2	IL-4	G/M-CSF
FDC-P1	+	765 ± 399ⁱ	46,619 ± 5,355	29,166 ± 4,948	243 ± 121	1,507 ± 204	10,749 ± 2,903	24,978 ± 4,083
WEHI-3 CM (10)	−	72 ± 35	19,798 ± 2,098	13,254 ± 3,093	51 ± 19	171 ± 89	1,248 ± 251	1,764 ± 270
FDC-P1	+	169 ± 77	32,404 ± 4,904	30,436 ± 7,741	98 ± 35	193 ± 64	13,767 ± 4,420	11,001 ± 2,508
IL-3 (6)	−	84 ± 45	24,228 ± 5,248	21,276 ± 5,772	33 ± 3	34 ± 3	885 ± 336	407 ± 74
FL5.12	+	612 ± 302	15,063 ± 1,707	9,360 ± 3,464	640 ± 250	720 ± 265	498 ± 211	667 ± 249
WEHI-3 CM (6)	−	98 ± 35	7,408 ± 2,512	8,182 ± 4,611	347 ± 219	364 ± 219	344 ± 278	282 ± 163
FL5.12	+	532 ± 60	6,148 ± 2,060	6,689 ± 2,656	432 ± 67	508 ± 154	541 ± 254	575 ± 37
IL-3 (3)	−	58 ± 5	6,040 ± 2,115	5,414 ± 1,944	96 ± 8	60 ± 20	64 ± 10	52 ± 9.7

* Either FDC-P1 or FL5.12 cells were maintained in continuous culture in the presence of 10% WEHI-3 CM or 20 U/ml purified IL-3. Numbers in parentheses represent the number of experiments performed.

‡ FDC-P1 or FL5.12 cells maintained in either WEHI-3 CM or purified IL-3 were harvested, washed four times in HBSS and replated at 2 × 10⁵ cells/ml in the presence or absence of factor for 24 h. The cells were then harvested and washed, and live cells were obtained. The cells were then plated at 10⁴ cells/well for an additional 24 h in the presence of various factors.

§ Cells were plated in the presence of medium alone, 10% WEHI-3 CM, 20 U/ml IL-3, 20 U/ml IL-1, 20 U/ml IL-2, 20 U/ml IL-4, or 20 U/ml of G/M-CSF for 24 h.

ⁱ Numbers represent cpm ± SEM of [³H]TdR incorporated during the last 4 h of culture.

factor concentrations (data not shown). In addition, similar patterns of [³H]TdR uptake were observed under serum-free conditions in the presence of WEHI-3 CM or after a 24-h factor starvation (data not shown).

Analysis of Factors that Cause a G_0 to G_1 Transition in IL-3-dependent Cell Lines. Resting (G_0) FDC-P1 cells or FL5.12 cells (Fig. 5) were cultured in the presence of IL-3, IL-4, G/M-CSF, or IL-1 for 12 or 24 h. At these time points, total cellular RNA content per cell was quantitated. We found that IL-3 induced an increase in total cellular RNA in both FDC-P1 and FL5.12 cells after 12 h of culture, indicating that a G_0 to G_1 transition had occured. By 24 h after IL-3 stimulation, both clones were actively cycling (Fig. 5). In FDC-P1 cells, IL-4 or G/M-CSF also caused a G_0 to G_1 transition by 12 h of culture. However, IL-4 or G/M-CSF did not promote entry of resting FDC-P1 cells into S, G_2, or M (Figs. 4B, 5). IL-1 (Fig. 5) and IL-2 (data not shown) had no effect on either FDC-P1 or FL5.12 cells at any time during the incubation.

While actively cycling FDC-P1 cells can enter the S phase in response to G/M-CSF or IL-4, we have shown that resting (G_0) cells are unable to enter the S phase in response to these factors (Table I; Fig. 4). This result suggests that IL-3 may provide an obligatory signal that enables cells to subsequently respond to G/M-CSF or IL-4. Therefore, we analyzed the kinetics of the appearance of responsiveness to these factors in resting (G_0) cells pulsed with IL-3 for various times, washed, and recultured in the presence of various CSFs for an additional 24 h. We found that a minimum of 12 h of incubation with IL-3 was required before responsiveness to G/M-CSF or IL-4 returned (Fig. 6). As a control, resting (G_0) cells preincubated in the presence of either G/M-CSF or IL-4 for 12 h, although able to enter G_1, were unable to enter S phase in response to either G/M-CSF or IL-4 (data not shown).

Both G/M-CSF and IL-4 Can Maintain Long-term Growth of FDC-P1 Cells. Stable, long-term cell lines have been established from FDC-P1 cells
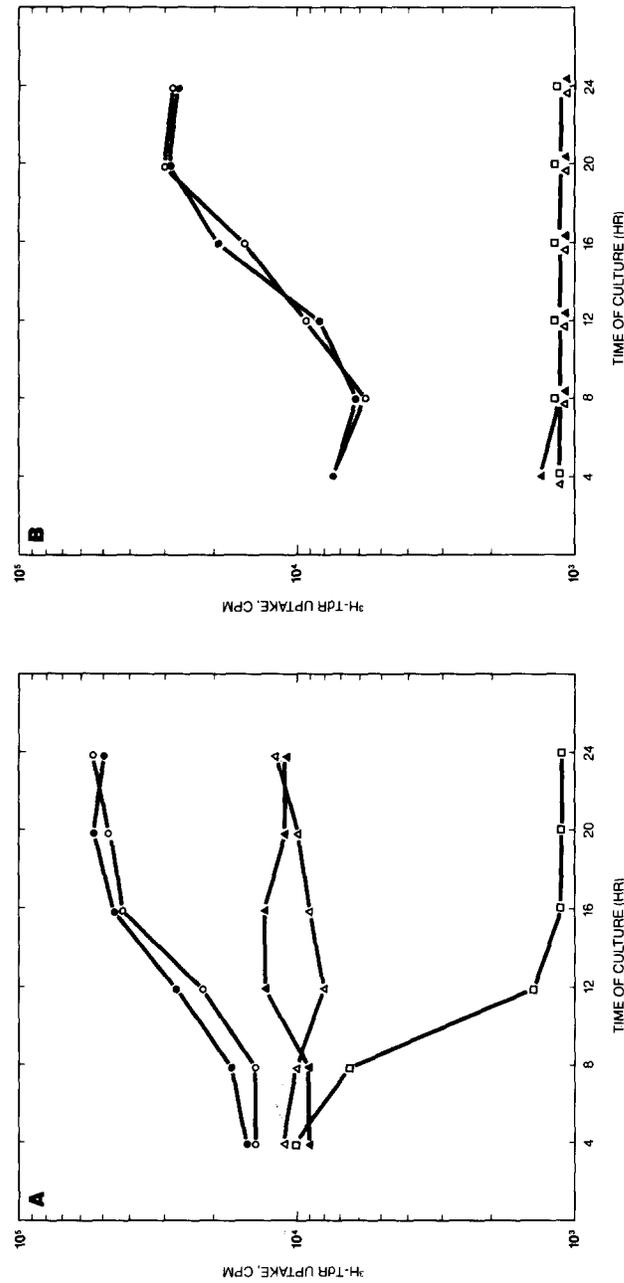
FIGURE 4. Kinetics of induction of [³H]TdR uptake in response to various CSF's. FDC-P1 cells, either actively cycling (A) or resting (G₀) (B) were plated in the presence of (●) WEHI-3 CM, (○) 20 U/ml IL-3, (△) 20 U/ml G/M-CSF, (▲) 20 U/ml IL-4, or (□) medium. [³H]TdR uptake was measured at the indicated timepoint. Data points represent the mean of triplicate cultures. The SEM was <15%.
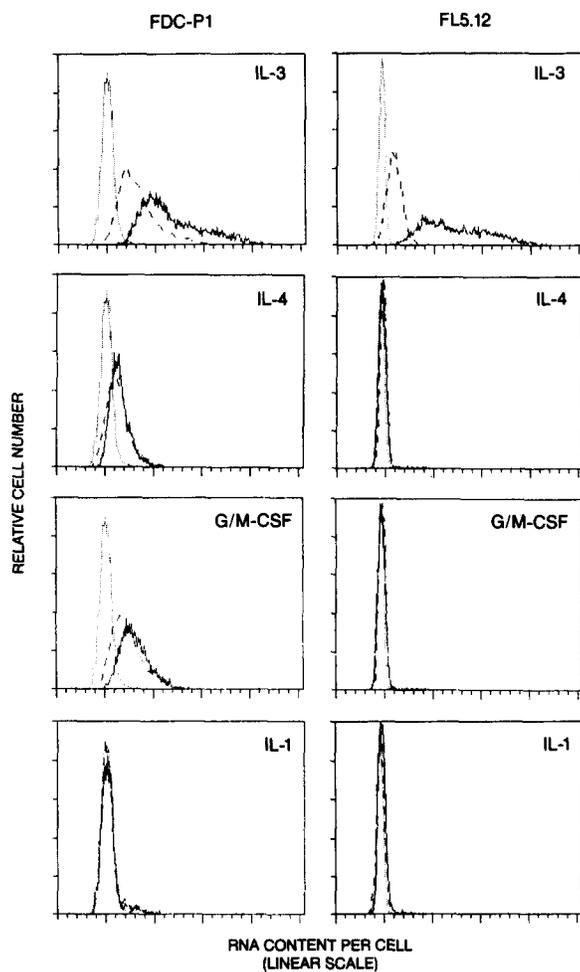
FDC-P1 FL5.12

FIGURE 5. Determination of a G_0 to G_1 transition induced by various CSFs in IL-3-dependent cell lines. Resting (G_0) FDC-P1 and FL5.12 cells were incubated in the presence of 20 U/ml IL-3, IL-4, G/M-CSF, or IL-1 for 12 (– – –) or 24 (———) h, after which acridine orange staining for RNA content was performed. (· · · ·) RNA content at time 0. y-axis, relative cells number; x-axis, fluorescence intensity, RNA content per cell.

RELATIVE CELL NUMBER

RNA CONTENT PER CELL
(LINEAR SCALE)

maintained in the presence of either G/M-CSF or IL-4. To determine whether these cells behave similarly to the cells from which they were derived, the lines were deprived of the respective CSF for 24 h. Unlike FDC-P1 cells maintained in either WEHI-3 CM or purified IL-3, FDC-P1 cells adapted for growth in either G/M-CSF or IL-4 did not make a complete transition into G_0. The majority of cells accumulated in G_1 (Fig. 7). This observation was also confirmed when CSF-deprived cells, restimulated with their respective ligand, quickly progress through G_1 and rapidly enter S, G_2 + M (assessed by [^{3}H]TdR uptake and flow cytometry) by 8–12 h of culture (not shown).

The finding that FDC-P1 cells maintained in either G/M-CSF or IL-4 were unable to enter G_0 upon factor removal showed that these cells had altered their growth pattern. We subsequently tested whether such cells might respond differently to other CSF than FDC-P1 cells maintained in IL-3. To investigate this possibility, a panel of CSF were analyzed for their ability to stimulate growth of either cycling or factor-deprived FDC-P1 cells maintained in either G/M-CSF or IL-4. We found that FDC-P1 cells maintained in G/M-CSF will uptake [^{3}H]-
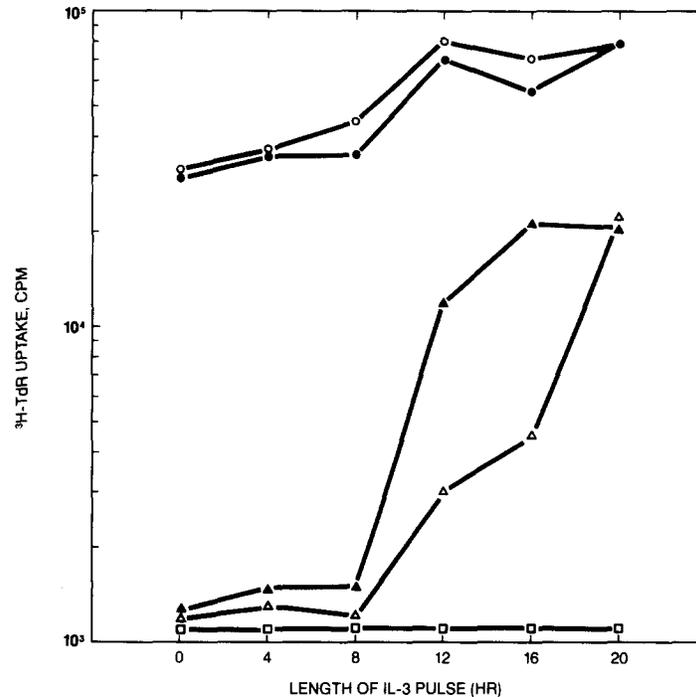
FIGURE 6.   Kinetics of induction of [³H]TdR uptake in response to G/M-CSF or IL-4 in resting (G₀) FDC-P1 cells. Resting (G₀) FDC-P1 cells were replated in the presence of 20 U/ml of IL-3 for the indicated time period, washed free of IL-3 and replated in the presence of (●) WEHI-3 CM, (○) 20 U/ml IL-3, (▲) 20 U/ml IL-4, (△) 20 U/ml G/M-CSF, or (□) medium for an additional 24 h. Data points represent the mean of triplicate cultures. The SEM was <15%.

TdR in response to WEHI-3 CM, IL-3, IL-4, or G/M-CSF with similar kinetics over a 24-h culture period whether these cells were actively cycling (Fig. 8A) or 24 h factor-deprived (Fig. 8B). Therefore, these cells behave differently than cells maintained in IL-3, which lose their ability to enter S phase in response to other CSF when factor-deprived (Fig. 4; Table I). FDC-P1 cells maintained in IL-4 will uptake [³H]TdR in response to either WEHI-3 CM, IL-3, or IL-4 whether or not they have been factor-deprived for 24 h (Fig. 8, C and D). However, unlike the cells from which they were derived, these cells lose their ability to respond to G/M-CSF. These results clearly demonstrate that FDC-P1 cells maintained in either G/M-CSF or IL-4 are altered in their growth factor responsiveness.

Discussion

The regulation of hematopoietic cellular growth and differentiation involves a complex set of interactions between hematopoietic stem cells and the microenvironment in which they reside. This process enables multipotential hematopoietic stem cells to generate all mature cells of the hematopoietic lineages. Stem cells are defined by their extensive proliferative capacity, their ability to differentiate along multiple lineages, and their ability to undergo self-renewal (27–
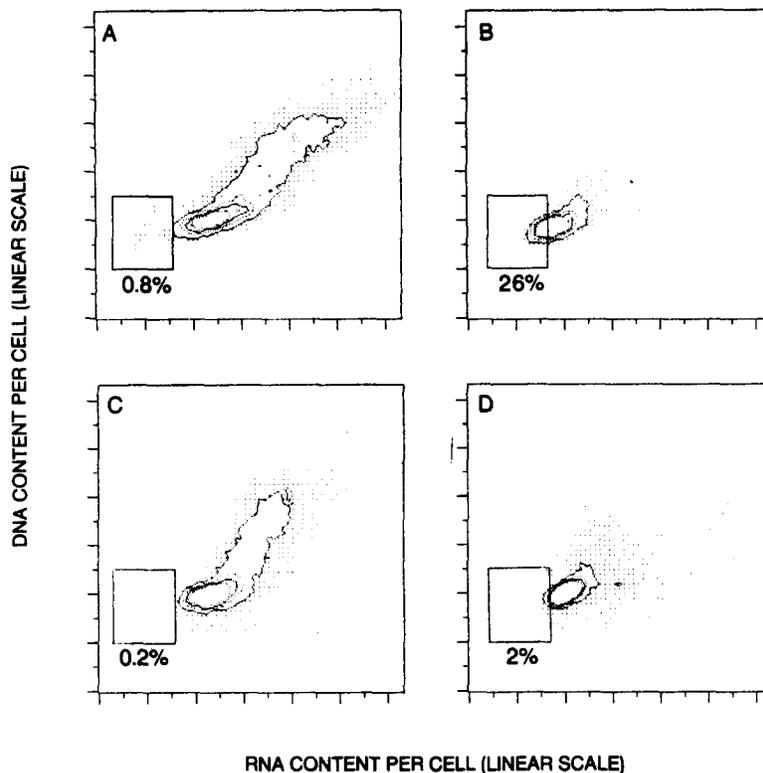
A

0.8%

B

26%

C

0.2%

D

2%

DNA CONTENT PER CELL (LINEAR SCALE)

RNA CONTENT PER CELL (LINEAR SCALE)

FIGURE 7.   Cell cycle compartments in FDC-P1 cells maintained in G/M-CSF or IL-4. FDC-P1 cells maintained in G/M-CSF (A and B) or IL-4 (C and D) were analyzed for DNA and RNA content using acridine orange. Either actively cycling (A and C) or 24-h factor-deprived (B and D) cells were used. x-axis, red fluorescence intensity, RNA content per cell; y-axis, green fluorescence intensity, DNA content per cell. Box designates G_0.

29). Presently, the mechanisms that regulate the growth and differentiation of these pluripotent stem cells are not completely understood.

Two major models have been proposed for the mechanism of stem cell renewal and commitment. The deterministic model of stem cell development suggests that the commitment of pluripotent hematopoietic stem cells to committed progenitors is regulated by the immediate microenvironment of the individual stem cell (30, 31). Appropriate external stimuli would act directly on stem cells to control commitment resulting in a uniform response to environmental stimuli. In contrast, the stochastic model of stem cell development predicts that multipotential stem cell differentiation is dependent upon different intrinsic probabilities that can be assigned to each of the hematopoietic lineages (32). These probabilities determine the likelihood of commitment to a specific lineage, with further commitment of progenitor cells being governed by progressive restriction in their differentiation potentials (33). External growth factors would determine which hematopoietic lineage can be derived from multipotential cells by influencing only the probability of such an event occurring within a self-renewing pool of stem cells rather than causing commitment of individual stem cells to a particular lineage (30, 34, 35). These growth factors have been classified as
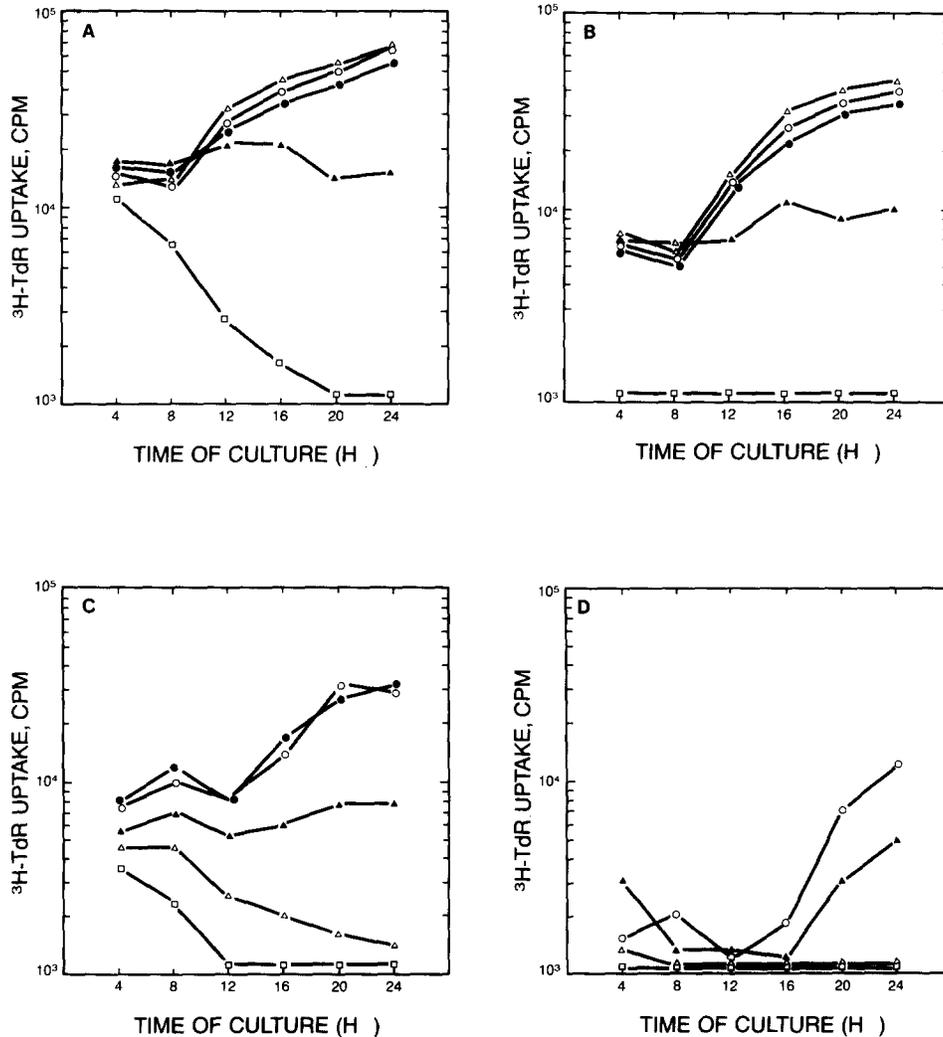
FIGURE 8. [³H]TdR uptake by FDC-P1 cells maintained in G/M-CSF or IL-4 in response to various CSFs. FDC-P1 cells maintained in G/M-CSF (A and B) or IL-4 (C and D), either actively cycling (A and C) or 24-h factor-starved (B and D), were analyzed for their response to (□) medium, (●) WEHI-3 CM, (○) 20 U/ml IL-3, (△) 20 U/ml G/M-CSF, or (▲) 20 U/ml IL-4 by [³H]TdR uptake. Data points represent the mean of triplicate cultures. The SEM was <15%.

either competence or progression factors, depending on the phase of the cell cycle where they are active (34). Competence factors might be regulators that act on cells in G_0 and allow them to enter G_1. Progression factors would influence cells after interaction with a competence factor and cause these cells to traverse into S, G_2, and M. Competence factors may regulate the cells responsiveness to a progression factor by up- or downregulating corresponding growth factor receptors or effecting an intrinsic change among cells recognizing such factors. Thus, it has been suggested that a hierarchy of growth factor responsiveness functionally controls different stages of maturation (36). This model implies that

as a cell matures, it loses its responsiveness to one factor and gains the ability to respond to another.

We have used two IL-3-dependent hematopoietic progenitor clones, FDC-P1 (a myeloid progenitor) and FL5.12 (a lymphoid progenitor), as a model for the regulation of cell cycle progression in hematopoietic stem cells. The metachromatic fluorochrome acridine orange was used to differentially stain single- versus double-stranded nucleic acids, thus identifying the various phases of the cell cycle. After selective denaturation of dsRNA, cellular DNA and RNA are stained differentially allowing for the discrimination between cells with different RNA content for a given DNA content (26, 37). It is possible to discriminate between cells in G_1, S, and G_2 + M phases of the cell cycle based on differences in their green flourescence (DNA content). Further, one can distinguish two qualitatively different compartments of G_1 according to RNA content, G_{1A} (early G_1) and G_{1B} (late G_1). The threshold dividing the A and B compartments represents the minimal RNA content of S phase cells, although the distribution of G_1 phase cells is unimodal with respect to RNA content (37). Actively cycling cells are fully separated from quiescent (G_0) cells based on total cellular RNA content. All quiescent cells have a 2N DNA content and very low RNA content, distinctly lower than G_{1A} cells, such that no overlap in RNA content is observed between G_0 cells and cells growing exponentially (37). We found that both FDC-P1 and FL5.12 cells, when deprived of IL-3, could be synchronized in the G_0 phase of the cell cycle. Accumulation of cells in the G_0 (resting) compartment was corroborated by direct measurement of RNA and DNA levels within single cells, background levels of both [^{3}H]TdR uptake and oxidative phosphorylation enzyme levels, as well as a significant decrease in cell volume. Furthermore, 24 h after factor deprivation, 50–70% of the cells remain viable. This finding indicates that the majority of cells, rather than a small subpopulation, reach G_0 before cell death occurs. Upon readdition of IL-3, these factor-deprived lines synchronously enter G_1 and ultimately progress through the S, G_2, and M phases of the cell cycle. Therefore, these results have clearly shown that IL-3 is a competence factor acting on a cell in G_0 and allowing it to progress into G_1. If exposure to IL-3 persists, it functions as a progression factor, allowing cells to traverse from G_1 into S, G_2, and M.

The pivotal role that IL-3 plays in hematopoietic development is shown by the ability of IL-3 to regulate responsiveness to other hematopoietic growth factors. This was demonstrated in FDC-P1 cells that were deprived of IL-3 and accumulated in G_0. These cells do not proliferate in response to G/M-CSF or IL-4. However, when they reenter G_1 in the presence of IL-3, growth in response to G/M-CSF or IL-4 is observed. These data would indicate that IL-3 can upregulate either the specific receptors for G/M-CSF or IL-4, or a closely related structure necessary for receptor signaling. These results are in contrast to those reported by Walker and coworkers (36). They suggest that the activity of IL-3 (multi-CSF) could be attributed to its ability to downregulate and thus activate other growth factor receptors. Since their experiments were performed using total bone marrow cells, the assignment of these different CSF receptors to a single class of cells was not possible. The use in our system of a clonal population of cells allows us to investigate ligand-receptor interactions on a clonal level. The generation

of a larger panel of hematopoietic progenitor cells, dependent upon IL-3 and/or other CSFs, as well as the development of receptor binding assays, will allow us to determine whether the responsiveness observed in FDC-P1 cells is a general phenomonen.

We have also found that either G/M-CSF or IL-4 can maintain long-term growth of FDC-P1 cells. In the presence of either G/M-CSF or IL-4, a striking change in the regulation of cell cycle progression occurs. Upon removal of factor, these cells accumulate in G_1 rather then G_0. This observation is corroborated by the functional ability of FDC-P1 cells maintained in G/M-CSF or IL-4 to proliferate in response to the same panel of CSFs that they respond to when not factor-deprived. In addition, FDC-P1 cells maintained in IL-4 are further distinguished by the loss of their ability to grow in response to G/M-CSF.

The significance of these findings in relationship to normal pluripotent stem cell development in vivo is corroborated by the recent finding (38) that most multipotent stem cells in vivo reside in the resting (G_0) state. Upon appropriate stimulation, these quiescent stem cells generate mature cells of all the hematopoietic lineages. In our system, CSF deprivation results in the accumulation of IL-3 dependent clones in G_0. However, these resting (G_0) cells still remain responsive to IL-3 and upon stimulation, enter G_1. At this point, cell growth can be initiated after stimulation with appropriate CSF. IL-3 can provide the necessary signal for $G_0 \rightarrow G_1$ transition and when sufficient levels of exogenous IL-3 are present, cells can transit from $G_1 \rightarrow M$ in 11–13 h. This pattern of growth can be maintained in vitro long term. Some IL-3-responsive cells (e.g., FDC-P1) can respond to other CSFs. However, cell growth supported by other CSFs could only be accomplished when cells reside in or beyond late G_1 or early S phase (8–12 h after IL-3 stimulation (Figs. 5 and 6). Maintenance of subsequent cell growth by other CSFs (G/M-CSF or IL-4) was not dependent upon exogenous IL-3 during G_1 phase and such cells did not transit to G_0 when depleted of G/M-CSF or IL-4. Loss of IL-3 dependence, failure to exit cell cycle to G_0, functional specialization, as well as striking changes in morphology and cell surface phenotype were observed when FDC-P1 cells were grown in G/M-CSF or IL-4.

The manner in which IL-3 regulates growth by supporting self-renewal and rendering some cells competent to long-term growth in response to additional CSFs provides strong clues to the biology of hematopoietic stem cells. Our in vitro model may mimic many features that result in assymetric division of stem cells (self-renewal coordinated with generation of differentiated progeny) and the ability of stem cells to reside for some time in G_0. Indeed, normal hematopoiesis may occur through the sequential utilization of different stem cell clones (38, 39). Recruitment of such clones from a "cryptic" or resting state and their subsequent differentiation program may be dependent upon the stage of cell cycle as well as sequential changes induced by CSF.

Summary

Hematopoietic cell development is regulated by a series of growth factors that are progressively restricted in their biological activity. IL-3 is a multi-lineage growth factor that supports the growth and differentiation of progenitor cells

belonging to multiple lineages. However, the mechanism by which IL-3 induces proliferation and differentiation of these cells is not completely understood.

In this report, we have used two IL-3-dependent cell lines, FDC-P1 (a myeloid progenitor) and F15.12 (a lymphoid progenitor) to investigate IL-3-mediated growth and differentiation. When either FDC-P1 or FL5.12 cells are deprived of IL-3, >90% of all cells accumulate in the G_0 phase of the cell cycle. Upon readdition of IL-3, the cells will reenter the active phases of the cell cycle. Therefore, IL-3 can act as both a competence ($G_0 \rightarrow G_1$) factor, and a progression ($G_1 \rightarrow M$) factor for hematopoietic precursor clones.

FDC-P1 cells can also proliferate in response to granulocyte/macrophage colony–stimulating factor (G/M-CSF) and IL-4 (B cell stimulatory factor 1 [BSF-1]). However, resting (G_0) FDC-P1 cells have lost their ability to grow in response to both G/M-CSF and IL-4, even though both factors can induce a $G_0 \rightarrow G_1$ transition. Therefore, G/M-CSF or IL-4 behave as progression factors among certain IL-3-responsive clones, and in those cases only in defined points in the cell cycle.

Both IL-4 and G/M-CSF can maintain long-term growth of FDC-P1 cells. Upon removal of factor for 24 h, these clones accumulate in the G_1 phase of the cell cycle and do not appear to enter G_0 even after 36 h of factor deprivation. Therefore, cells maintained in G/M-CSF or IL-4 have altered growth requirements compared with the IL-3-dependent lines from which they were derived. The ability of various hematopoietic growth factors to regulate cell cycle progression in IL-3-dependent cell lines is dependent not only upon the lineage from which these cells were derived, but also the phase of the cell cycle in which those cells reside. The consequences of these interactions dictate the manner by which various clones will respond to CSFs and whether the cells will grow and/or differentiate.

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