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Interleukin-6- and Leukemia Inhibitory Factor-Induced Terminal Differentiation of Myeloid Leukemia Cells Is Blocked at an Intermediate Stage by Constitutive c-*myc*

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Interleukin-6 (IL-6) and leukemia inhibitory factor (LIF), two multifunctional cytokines, recently have been identified as physiological inducers of hematopoietic cell differentiation which also induce terminal differentiation and growth arrest of the myeloblastic leukemic M1 cell line. In this work, it is shown that c-*myc* exhibited a unique pattern of expression upon induction of M1 terminal differentiation by LIF or IL-6, with an early transient increase followed by a decrease to control levels by 12 h and no detectable c-*myc* mRNA by 1 day; in contrast, c-*myb* expression was rapidly suppressed, with no detectable c-*myb* mRNA by 12 h. Vectors containing the c-*myc* gene under control of the β-actin gene promoter were transfected into M1 cells to obtain M1myc cell lines which constitutively synthesized c-*myc*. Deregulated and continued expression of c-*myc* blocked terminal differentiation induced by IL-6 or LIF at an intermediate stage in the progression from immature blasts to mature macrophages, precisely at the point in time when c-*myc* is normally suppressed, leading to intermediate-stage myeloid cells which continued to proliferate in the absence of c-*myb* expression.

Terminal differentiation of eucaryotic cells involves the regulated progression of cells through successive stages of differentiation and growth inhibition, ultimately resulting in growth arrest. A profound example of this process, which continues throughout life, is the complex formation of blood cells (44). The establishment of in vitro culture systems for the clonal development of bone marrow cells (6, 34) and the availability of the M1 myeloid leukemia cell line, which can be induced for differentiation (M1D+) by physiological myelopoietic differentiation inducers (38), provide an excellent biological system with which to study the molecular biology of normal cell growth and differentiation, and lesions that afflict it, leading to oncogenesis and its progression (18, 19, 25, 26). Recently it has been shown that interleukin-6 (IL-6) and leukemia inhibitory factor (LIF), two multifunctional cytokines (2, 43, 45), also act as hematopoietic differentiation inducers which induce terminal differentiation and growth arrest of M1 cells (15, 41).

The c-*myc* proto-oncogene is expressed in almost all proliferating normal cell types and is down-regulated in many cell types when they are induced to terminally differentiate, and abnormal expression has been associated with many naturally occurring neoplasms (9, 21). However, the function of the c-*myc* protein remains controversial (11). We have shown that the expression of c-*myc*, which is high in both normal and M1 leukemic myeloid precursor cells during cell proliferation, is suppressed following induction of terminal differentiation and growth arrest by conditioned medium of mouse lungs (LUCM) (24), a potent physiological source of hematopoietic growth and differentiation inducers that has recently been shown to contain LIF and IL-6 (16a, 41). Forced expression of c-*myc* in several cell types, including preadipocyte, erythroleukemia, myogenic, and embryonic F9 teratocarcinoma cells, which are induced for differentiation by either undefined (low serum) or chemical inducers, was shown to block differentiation in some cells (10, 12, 14, 35) but not in others (32, 40). Whether these differing results reflect the type of nonphysiological inducer used or variability in the role of c-*myc* in different cell types is not clear.

The availability of the M1 myeloblastic leukemia cells that can be induced for terminal differentiation and growth arrest by LIF or IL-6, physiological inducers of myeloid differentiation, in conjunction with the multitude of immediate-early to late genetic and morphological markers that have been identified to be associated with the different stages of myeloid maturation (24, 28, 29, 31), provides a unique opportunity both to better understand the molecular controls of IL-6- and LIF-induced terminal differentiation and to ascertain the role of c-*myc* suppression in this process. In this work, vectors containing the c-*myc* gene under control of the β-actin gene promoter have been transfected into M1 cells to obtain M1myc cell lines which constitutively synthesized c-*myc*. Our results show that c-*myc* exhibited a unique pattern of expression upon induction of M1 terminal differentiation by LIF or IL-6 and that deregulated and continued expression of c-*myc* inhibited terminal differentiation. Differentiation was blocked at an intermediate stage in the progression from immature blasts to mature macrophages, resulting in intermediate-stage myeloid cells which continued to proliferate in the absence of c-*myb* expression.

MATERIALS AND METHODS

Cells and cell culture. The murine M1 myeloid leukemic cell line was obtained from E. R. Stanley (Albert Einstein College of Medicine). It was recloned in soft agar, and clones were tested for differentiation-associated properties as described previously (27, 28). M1 differentiation-competent clone 6 (M1D+) (24, 28), induced for differentiation by IL-6 or LIF, was used in this study. Cells were cultured as described previously (27, 28). Viable cell numbers were

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determined by trypan blue dye exclusion and counted in a hemacytometer.

Cytokines and other compounds used. Serum-free LUCM was prepared with LiCl (20) and used at a concentration of 10%. Purified human recombinant IL-6 (0.5 mg/ml) was a gift from L. Souza, Amgen, Inc., Thousand Oaks, Calif., and was used at a concentration of 100 ng/ml. Murine recombinant LIF in Cos cell conditioned medium was prepared by using the expression vector pcD-LIF (F. Lee, DNAX) and was used at a concentration of 10 U/ml (27). The cytokines were titrated for differentiation-inducing and growth inhibitory activities as described previously (24, 27, 28). Optimum concentrations of human recombinant IL-6 and murine recombinant IL-6 gave identical responses with M1D+ cells, and purified human recombinant IL-6 was used in the experiments.

Assays for differentiation-associated properties. Fc and C3 receptors (27, 30), lysozyme activity in cell extracts and in the growth medium (22, 27), and cell attachment (27) were determined as described previously. Morphological differentiation was determined by counting at least 300 cells on May-Grünwald-Giemsa-stained cytospin smears and scoring the proportion of immature blast cells, cells at intermediate monocyte stages of differentiation, and mature macrophages (27, 28).

Flow cytometry. Samples of 2×10^6 cells were stained with propidium iodide (Calbiochem) at a final concentration of 50 μg/ml in the presence of 0.1% Triton X-100 and 90 U of RNase (Worthington) per ml. Cells were analyzed in a fluorescence-activated cell sorter (Becton Dickinson FACS IV).

General recombinant DNA techniques and DNA probes. Plasmid preparations, restriction enzyme digestions, DNA fragment preparations, and agarose gel electrophoresis were as described before (24, 27, 28). Probes for c-*myc*, c-*myb*, and the β-actin gene were the same as used previously (24). *junB* (*MyD21*) and *MyD88* probes were cDNAs cloned in this laboratory (28, 29). Murine ferritin light chain was also cloned in this laboratory from a cDNA library of myeloid-enriched bone marrow, sequenced by S. Suggs (Amgen), and found to match the known sequence (3). DNA for probes was labeled by random priming to a specific activity equal to or greater than 10^9 cpm/μg (13).

Construction of pAc.Myc. The 1.5-kb *Sst*I-*Hin*dIII restriction fragment containing 60 bp of untranslated region and all of the coding region (exons 2 and 3) of a mouse c-*myc* cDNA (33) was cloned into the *Hin*dIII site of pHb APr-1-neo (17) so that c-*myc* was under control of the β-actin gene promoter. The *Sst*I and *Hin*dIII sites were blunt ended with T4 polymerase (IBI).

DNA transfections. Transfections were performed by electroporation (Bio-Rad Gene Pulsor). A pulse was delivered to a 0.7-ml suspension containing 1.5×10^7 cells and 50 μg of linearized plasmid DNA. The cells were appropriately diluted and after 48 h were subjected to selection in growth medium containing 400 μg of geneticin (G418 sulfate; GIBCO) per ml. After 4 weeks, surviving cells were diluted and then cloned in agar in the presence of geneticin. Transfectants were maintained in 200 μg of drug per ml.

Genomic DNA extraction, Southern blotting, and hybridization. DNA was extracted as described previously (39). Following the appropriate restriction enzyme digestions, 20 μg of DNA was size fractionated on a 1% agarose gel and blotted onto Gene Screen Plus according to recommended procedures (NEN). Filters were prehybridized in hybridization buffer (10% dextran sulfate, 1 M NaCl, 1% sodium dodecyl sulfate) at 65°C for 2 to 3 h and, following removal of the buffer, hybridized at 65°C for 12 to 16 h in additional buffer containing denatured probe at 2×10^6 cpm/ml and sheared salmon sperm DNA at 100 μg/ml. Blots were washed according to recommended procedures for Gene Screen Plus (NEN) and exposed to X-ray film at −80°C.

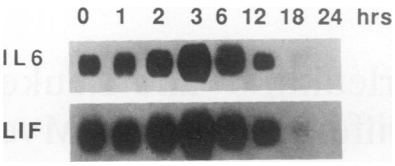

FIG. 1. Detailed time course of c-*myc* RNA expression in M1 cells following induction of differentiation by IL-6 or LIF. Northern blots of RNA, isolated following treatment with IL-6 or LIF for the designated times, were hybridized to a c-*myc* probe.

RNA extraction, Northern (RNA) blotting and hybridization. RNA was extracted by the method of Chomczynski and Sacchi (7), using guanidinium thiocyanate. Total RNA (5 μg/lane) was electrophoresed on 1% agarose-formaldehyde gels. Preparation of Northern blots, hybridization and washing conditions, and stripping blots of probe to rehybridize were done as described previously (27). For quantitation of individual mRNAs, films were exposed for periods during which band intensity was linear with respect to time, and relative intensities of hybridization signals were measured at 560 nm with the gel scan program of a Beckman DU7 spectrophotometer.

RESULTS

c-*myc* expression analysis in M1 following IL-6 or LIF induction of terminal differentiation and growth arrest. In a previous study, we showed that c-*myc* transcripts are barely detectable 1 day following induction of terminal differentiation of M1 myeloid leukemia cells, using LUCM (24). Since in this study we used IL-6 and LIF to induce differentiation, it was necessary to ascertain whether c-*myc* expression was down-regulated with these cytokines as well. Also, to analyze the effects of deregulated and constitutive expression of c-*myc* expression on IL-6- or LIF-induced myeloid differentiation and growth arrest, it was necessary to examine c-*myc* RNA levels at earlier times. In exponentially growing M1 cells treated with either IL-6 or LIF, steady-state levels of c-*myc* transcripts increased rapidly and transiently (Fig. 1). Expression peaked by 3 h at a level 10 times that of the control and gradually declined thereafter (Fig. 1). By 12 h the level of c-*myc* was similar to that of untreated M1 control cells, and by 1 day c-*myc* was no longer detectable.

Construction and analysis of M1myc cell lines constitutively expressing c-*myc* RNA. The vector pAc.Myc (Fig. 2A), in which the coding region of c-*myc* is under control of the β-actin gene promoter (see Materials and Methods), was transfected via electroporation into the recipient myeloid leukemic M1 cell line. Transfectants (M1myc) were selected for resistance, conferred by the Neo^r gene, to the drug geneticin. In addition, transfectants obtained by using the vector without the c-*myc* insert (M1neo) and selected for resistance to geneticin also were isolated as controls. Ten M1myc clones and five M1neo clones were isolated and established as clonal cell lines.

Southern blot analysis of genomic DNA showed two *myc*-hybridizing bands for clone M1myc2 (Fig. 2B), one corresponding to the endogenous c-*myc* gene and the other

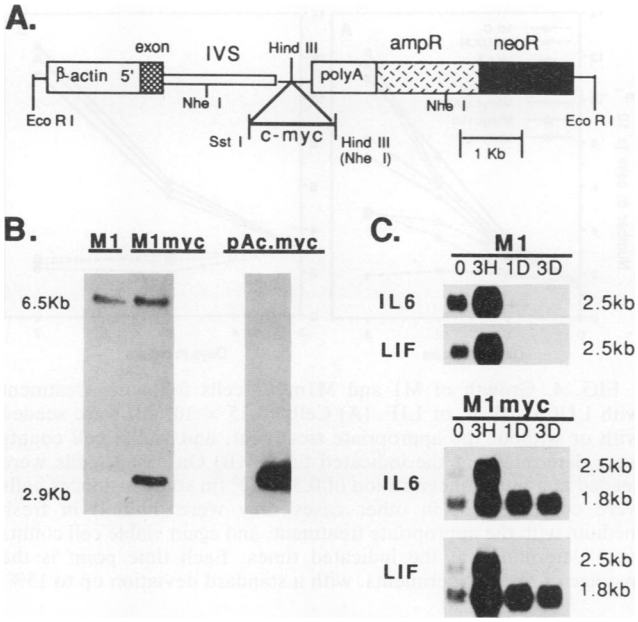

FIG. 2. (A) pAc.Myc vector used for transfection. For construction, see Materials and Methods. (B) Southern blot analysis of genomic DNA demonstrating that M1myc2 (M1 transfected with pAC.myc) contains both the endogenous and exogenous c-*myc* genes. For the analysis, 20 μg of genomic DNA and 0.1 μg of pAC.myc were digested with *Nco*I, resolved on a 1% agarose gel, transferred to Gene Screen Plus (NEN), and hybridized to a c-*myc* DNA fragment. (C) Analysis of c-*myc* RNA expression in M1 and M1myc2 cells before and after treatment with IL-6 and LIF at the indicated times. Northern blots were hybridized to a c-*myc* fragment.

corresponding to the exogenous c-*myc* gene. Similar results were obtained for all M1myc cell lines that expressed exogenous c-*myc* mRNA.

Cloned M1myc cell lines were analyzed for the expression of exogenous c-*myc* transcripts, readily distinguished from endogenous transcripts by size. Northern blot analysis of M1myc2 RNA clearly showed the presence of both c-*myc* transcripts (Fig. 2C). For four representative M1myc cell lines, including M1myc2, the exogenous transcript levels were considerably higher than endogenous levels; however, for each cell line the steady-state level of total c-*myc* transcripts was similar to that in M1 control cells, since the endogenous level was lower than the M1 control level (Fig. 2C). Thus, high expression of the exogenous gene leads to down-regulation of the endogenous c-*myc* gene, in contrast to results obtained with murine erythroleukemia cells (10). In six other M1myc cell lines, the exogenous c-*myc* transcript levels were lower than endogenous c-*myc* levels.

Analysis of RNA from M1myc2 cells before and at various times after treatment with IL-6 and LIF demonstrated that endogenous c-*myc* was down-regulated just like for M1 cells; however, the steady-state levels of exogenous c-*myc* transcripts remained high (Fig. 2C). In M1myc2 cells both the endogenous and exogenous c-*myc* transcripts were elevated by 3 h following treatment with either IL-6 or LIF, and the steady-state level of exogenous c-*myc* remained higher than that of untreated M1myc cells for at least 3 days (Fig. 2C). Therefore, c-*myc* was constitutively expressed in M1myc2 cells. For all other M1myc cell lines studied, the results were similar to what has been shown for M1myc2.

Constitutive, elevated expression of c-*myc* and terminal myeloid differentiation. We wanted to ascertain whether constitutive, elevated levels of c-*myc* expression affected the induction of terminal myeloid differentiation using physiological inducers. In addition to IL-6 and LIF, LUCM was used, although it is not completely defined, because its inducing effects are stronger and the intent was to challenge the M1myc cells to differentiate with the most potent inducers available. Detailed results are presented for M1myc2, which expressed elevated levels of c-*myc*, and compared with results for the parental M1 cell line. Five M1neo cell lines gave results similar to those for M1; three M1myc cell lines, which expressed high levels of exogenous c-*myc* mRNA, behaved similarly to M1myc2.

In contrast to the results obtained with the parental M1 cell line, there were no mature cells present in the M1myc2 cell line following stimulation with either LUCM, IL-6, or LIF (Table 1); 65 to 75% of the cells resembled cells at an intermediate stage of differentiation, and the remainder were blasts. It is clear that even with LUCM the M1myc2 cells did not undergo terminal differentiation. The difference in the response to differentiation factors of M1 compared with M1myc2 can be readily seen by examining the cell morphology 4 days after treatment with IL-6 (Fig. 3a to d). Similar results were seen with LIF.

Analysis of M1myc cell lines which constitutively expressed low levels of c-*myc* also showed no induction of mature cells following treatment with IL-6, LIF, or LUCM

TABLE 1. Induction of terminal differentiation, growth inhibition and differentiation-associated properties by LUCM, IL-6, and LIF in parental M1 and M1myc2 cells

Clone	Inducer[a]	Cell no.[b] (10^6)	Cell adherence[b] (%)	Cells in G_0/G_1[c] (%)	Fc receptor[b] (%)	C3 receptor[b] (%)	Lysozyme[b,d]	Cell type (%)[b]		
								Blast	Intermediate	Mature
M1	None	2.1	<1	48.2	0.5	1.2	0.13	>99	<1	0
	LUCM	0.22	89	64.8	42	63	11.8	3	23	74
	IL6	0.39	67	63.4	35	56	8.9	9	57	34
	LIF	0.38	63	63.2	33	57	7.9	10	59	31
M1myc2	None	2.3	<1	40.4	0.4	1.4	0.11	>99	<1	0
	LUCM	1.2	8	41.6	40	19.3	0.39	28	72	0
	IL6	1.5	6	42.9	37	17.1	0.42	31	69	0
	LIF	1.3	4	41.1	32	11.8	0.46	34	66	0

[a] LUCM was used at a concentration of 10% (vol/vol), IL-6 was used at 100 ng/ml, and LIF was used at 10 U/ml. Cells were seeded at a density of 0.1×10^6.
[b] Determined 4 days after the cells were seeded. All values represent the mean of three independent determinations with standard deviations up to 15%.
[c] Cytofluorometric analysis was performed after 3 days (see Materials and Methods), and the percentages of cells in G_0/G_1, S, and G_2/M were ascertained.
[d] Given in microgram equivalents per 5×10^6 cells.

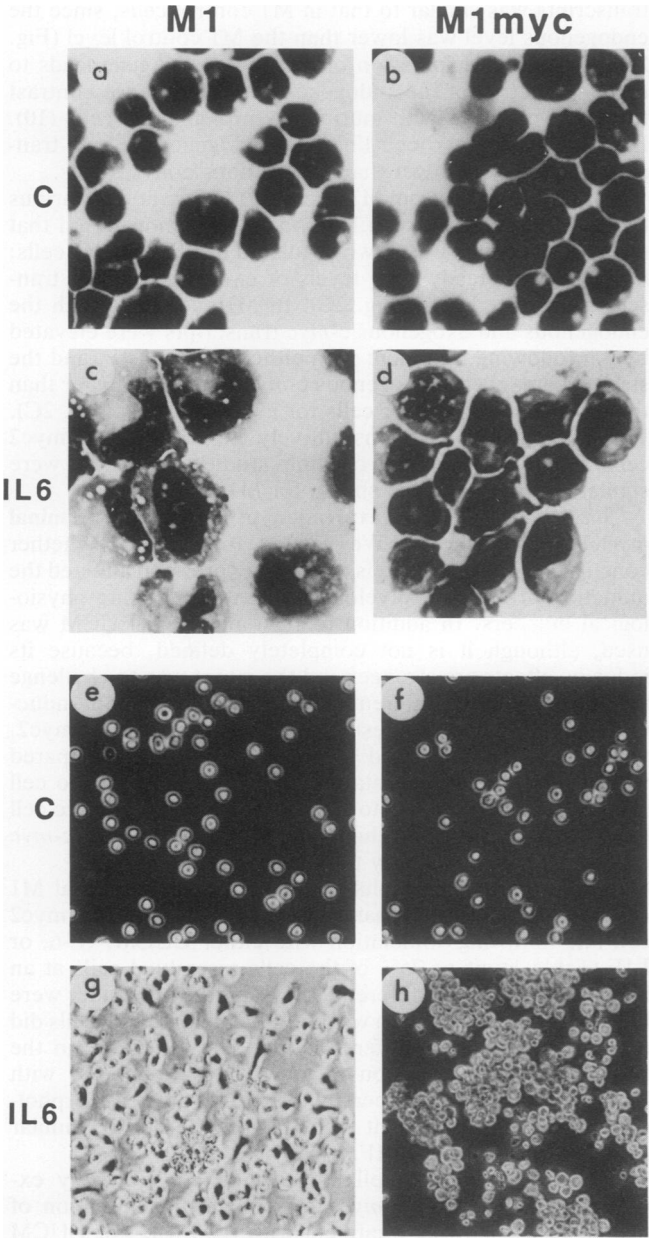

FIG. 3. Comparison of M1 and M1myc cells following treatment with IL-6. Shown are photomicrographs of May-Grünwald-Giemsa-stained cytospin smears of M1 and M1myc2 cells without (a and b) and with (c and d) IL-6 for 4 days (magnification, ×420). Blasts are characterized by scant cytoplasm and round or oval nuclei; mature cells have large amounts of cytoplasm, irregularly shaped nuclei, and vacuoles. (e to h) M1 and M1myc cells in culture without (e and f) and with (g and h) IL-6 for 4 days (magnification, ×90).

except for M1myc9, which resulted in 2% mature cells. Thus, even continued expression of low levels of c-*myc* blocked terminal differentiation.

By 1 day following induction for myeloid differentiation, many of the M1 cells formed aggregates and a few (5%) of the cells adhered to the tissue culture dish. By day 2, most of the aggregates had disassociated and a larger percentage (30 to 50%) of the cells were adhering. By 4 days, the majority of the M1 cells were adhering to the tissue culture dish (Table 1). In contrast, for M1myc2 very few cells were sticking to the culture dish (Table 1), and many of the cells were arranged in large aggregates (Fig. 3e to h show cells treated with IL-6; similar results were obtained with LIF). This is further evidence that M1myc2 cells treated with physiological inducers for myeloid differentiation were blocked at an intermediate stage of differentiation.

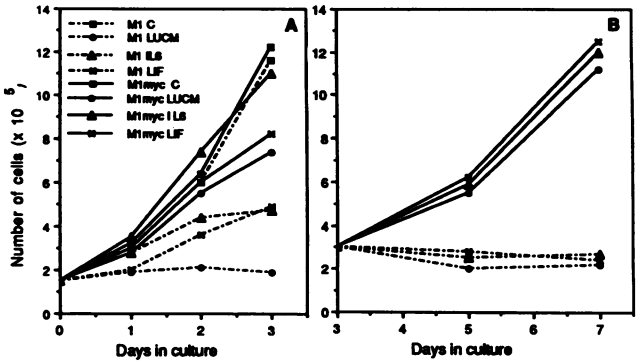

FIG. 4. Growth of M1 and M1myc2 cells following treatment with LUCM, IL-6, or LIF. (A) Cells (0.15×10^6/ml) were seeded with or without the appropriate treatment, and viable cell counts were determined at the indicated times. (B) On day 3, cells were seeded at a final concentration of 0.3×10^6 (in some instances cells were concentrated; in other cases they were diluted) in fresh medium with the appropriate treatment, and again viable cell counts were determined at the indicated times. Each time point is the average of three experiments, with a standard deviation up to 15%.

Effects of constitutive, elevated expression of c-*myc* on growth arrest associated with terminal differentiation and of withdrawal from the cell cycle. Myeloid leukemic M1 cells induced for terminal differentiation by LUCM, IL-6, or LIF became growth arrested (Table 1). By 4 days following stimulation with LUCM, IL-6, or LIF, the cell concentration was much higher for M1myc2 than for the parental M1 cell line (Table 1), although it was lower than for cells not induced to differentiate. To determine whether M1myc2 was growth arrested at a later time than M1 or whether M1myc2 continued to proliferate in the presence of differentiation inducers, growth kinetics was analyzed (Fig. 4). The kinetic studies clearly demonstrated that M1myc2 continued to proliferate for at least 7 days in the presence of LUCM, IL-6 or LIF, when M1 no longer proliferated, and the doubling time was 2 days, compared with 20 h for untreated M1myc or M1. Clearly, continued expression of c-*myc* reversed the growth arrest normally induced by LUCM, IL-6, and LIF.

Withdrawal from the cell cycle is believed to be a prerequisite for terminal differentiation of most cell types (14). M1 myeloid leukemic cells accumulated in the G_0/G_1 phase at the expense of the other cell cycle phases when induced to differentiate; in contrast, M1myc2 cells treated with inducers of myeloid differentiation behaved like untreated M1myc2 cells (Table 1). Our results are consistent with the notion that enforced expression of c-*myc* precluded cells from entering into a specific growth-arrested state in G_0/G_1.

Expression of the proto-oncogene c-*myb* in M1 and M1myc cells. The proto-oncogene c-*myb*, whose transcripts are found primarily in tissues of hematopoietic origin (4, 16), was expressed in autonomously proliferating M1 cells and was down-regulated when M1 was induced to terminally differentiate by LUCM (24), IL-6 (Fig. 5), or LIF (data not shown). By 3 h the steady-state level of c-*myb* had dropped, and it was barely detectable by 12 h (Fig. 5).

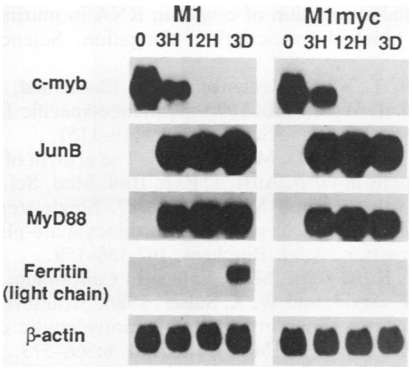

FIG. 5. Analysis of expression of c-*myb*, *junB*, *MyD88*, and the ferritin light-chain gene following IL-6 treatment of M1 and M1myc2 cells. Expression was analyzed by hybridization to Northern blots, using total RNA extracted from cells at the indicated times.

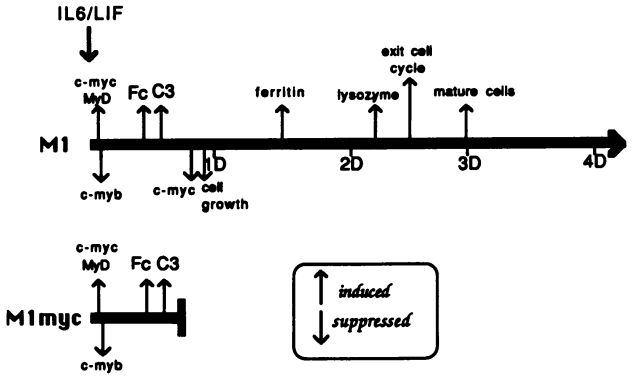

FIG. 6. Schematic representation of induction of M1 differentiation and the block in M1myc2 following treatment with IL-6 or LIF.

Since c-*myb* expression is associated with proliferating myeloid cells and early suppression follows induction of M1 myeloid cell differentiation and growth arrest, it was interesting to ascertain whether c-*myb* was suppressed and continued to remain suppressed in M1myc2 cells following stimulation with myeloid differentiation factors, which results in proliferating intermediate-stage myeloid cells. The expression of c-*myb* was suppressed in M1myc2 cells, the same as for M1 cells (Fig. 5), at both early and late times following treatment with IL-6.

Induction of primary response genes and changes in gene expression associated with later stages of differentiation. Immediate-early response genes (*MyD*), which are induced within 30 min and in the absence of protein synthesis following stimulation of M1 cells with LUCM or IL-6, have been isolated in this laboratory (28, 29). Many of these genes continue to be expressed for at least 3 days. We wanted to ascertain whether any *MyD* genes were induced in M1myc2 cells following stimulation with a myeloid differentiation factor and whether expression was maintained as for M1 cells. The two genes selected were *junB*, originally cloned as *MyD21*, and *MyD88*, a novel gene whose sequence has been reported recently (29). Both *junB* and *MyD88* were strongly induced and continued to be expressed both in M1 and M1myc2 cells following stimulation with IL-6 (Fig. 5). That these genes continued to be expressed at the same level in both M1 and M1myc2 cells is consistent with the notion that M1myc2 cells treated with factors which induced terminal differentiation of M1 cells were blocked at an intermediate stage of differentiation and did not revert to the blast stage. The expression of other *MyD* genes, namely, c-*jun* and *MyD126* (28), was also found to be induced, and the genes were expressed normally in IL-6-treated M1myc2 cells (data not shown).

Fc and C3 receptors start to appear about 12 h following induction of differentiation of M1 cells and continue to increase (30). The percentage of cells with Fc receptors were similar for M1 and M1myc2 cells; however, C3 receptors were significantly lower for M1myc2 cells (Table 1).

It has been reported that ferritin light-chain mRNA is induced in the human myeloid cell line HL60 (8). Here we show that M1 cells induced for differentiation with IL-6 also induced increased levels of ferritin light-chain transcripts (Fig. 5), with high expression by 3 days. Clearly, no such induction occurred in M1myc2 cells.

Lysozyme is induced in M1 cells, in which it is detected as early as 2 days following stimulation for terminal differentiation and continues to increase (22; Table 1). However, no lysozyme was detected for M1myc2 cells induced for differentiation (Table 1).

These results clearly corroborated the previous results showing that M1myc2 cells, which constitutively expressed high levels of c-*myc* transcripts, were blocked at an intermediate stage of differentiation following stimulation by LUCM, IL-6 or LIF, all of which induced terminal differentiation and growth arrest of the parental M1 and transfected M1neo cell lines.

DISCUSSION

In this report we have shown that terminal myeloid differentiation induced by IL-6 or LIF required suppression of c-*myc* expression. Continuous expression of c-*myc* blocked IL-6- or LIF-induced terminal differentiation of M1 myeloid leukemia cells at an intermediate stage in the progression from immature blasts to mature macrophages, shown both by cell morphology and by using genetic markers (Fig. 6). No mature macrophages were detected, but many of the cells were arrested at an intermediate stage of monocyte differentiation. Immediate-early genes (*junB*, c-*jun*, *MyD126*, and *MyD88* have been tested) were induced, c-*myb* was down-regulated, Fc receptors were induced, low levels of C3 receptors were induced, and both ferritin light chain and lysozyme, which are activated late during differentiation, were not induced. In addition, the cells were precluded from exiting the cell cycle. Growth arrest associated with terminal myeloid differentiation was reversed; however, the doubling time for M1myc2 cells in the presence of IL-6 or LIF was 2 days, compared with 20 h for untreated M1 or M1myc cells. The block in myeloid differentiation caused by continuous and elevated expression of c-*myc* is consistent with the time that c-*myc* is normally suppressed in IL-6- or LIF-treated M1 cells (Fig. 6).

Continued expression of c-*myc* clearly exerts a pleiotropic effect on myeloid differentiation. By the time c-*myc* is normally suppressed following treatment with IL-6 or LIF, both M1 and M1myc cells have undergone a multitude of changes. Although all these changes have taken place, the failure to suppress c-*myc* expression still results in a block to differentiation. Experimental evidence that c-*myc* binds to DNA and may activate or suppress the expression of a variety of genes is highly suggestive (5, 11, 21, 42). If this is

the case, then continued expression of c-*myc* may prevent the suppression or activation of genes necessary for terminal myeloid differentiation and growth arrest.

We report, for the first time, that myeloid cells proliferated, albeit at a reduced rate, when no c-*myb* gene product was available. Usually, c-*myb* expression is associated with proliferating cells and inhibition of c-*myb* expression is associated with the terminally differentiated and growth-arrested state (23, 24, 36). Consistent with this notion, c-*myb* continued to be expressed when constitutive c-*myc* blocked chemically induced differentiation of mouse erythroleukemic cells (11, 12). Therefore, the finding that c-*myb* was no longer expressed in IL-6- or LIF-treated M1myc2 cells, which were blocked at an intermediate stage of differentiation and continued to proliferate, was quite surprising.

The failure of M1myc to express the proto-oncogene c-*myb* following treatment with IL-6 or LIF may account for the observed reduced growth rate, consistent with the observation that antisense c-*myb* oligomers which diminish the level of c-*myb* protein also inhibit proliferation, to various degrees, of human myeloid leukemic cell lines (1). On the other hand, the reduced growth rate may be accounted for by the synthesis of beta interferon, which plays an autocrine role suggested to account for part of the growth inhibition associated with terminal myeloid differentiation (37) and which may inhibit cell proliferation independent of continued c-*myc* expression. Finally, the M1myc myeloblastic leukemia cell has undergone extensive changes in gene expression following treatment with IL-6 or LIF prior to sensing that c-*myc* expression is deregulated, and one or more of these changes may account for the slower growth rate.

Concluding remarks. Our results clearly demonstrated that deregulated and continuous expression of c-*myc* in M1 myeloid leukemic cells treated with the differentiation inducer IL-6 or LIF resulted in intermediate-stage myeloid cells which continued to proliferate in the absence of c-*myb* expression (Fig. 6). c-*myc* may have multifunctional roles in regulating myeloid differentiation and growth, as seen by the pleiotropic effects resulting from continuous expression of c-*myc*, which is usually suppressed at 18 h after induction of differentiation. In addition to the importance of c-*myc* suppression for myeloid differentiation, the very rapid initial increase in c-*myc* expression may play a role as well.

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