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A System for Monocytic Differentiation of Leukemic Cells HL 60 by a Short Exposure to 1,25-Dihydroxycholecalciferol (42098)

GEORGE P. STUDZINSKI, AMARJIT K. BHANDAL, AND ZAMIR S. BRELVI

Department of Pathology, UMDNJ-New Jersey Medical School, Newark, New Jersey 07103

Abstract. The human promyelocytic cell line HL 60 can be induced to differentiate toward more mature myeloid or monocytic forms by a variety of agents. This process is thought to require several days of exposure to the inducer, thus making it difficult to identify the early cellular changes which are fundamental to the differentiation program, and to relate the induction to phases of the cell cycle. In order to study the kinetics of leukemic cell differentiation we have developed a system for the induction of rapid monocytic maturation in a subpopulation of HL 60 cells. The cells are exposed to 10^{-7} *M* 1,25-dihydroxycholecalciferol for 4 hr in serum-free medium. Subsequent incubation in a complete medium results in cellular differentiation recognizable by several criteria (phagocytosis, nonspecific esterase reaction, adherence to substratum, cell morphology) beginning at 10 hr from the exposure to the inducer. Approximately 20 hr later 30–40% of the cells in culture show the differentiated phenotype and are capable of phagocytosis. The proportion of differentiated cells in culture decreases thereafter. This system has been utilized to study the expression of c-myc oncogene in relation to the kinetics of maturation, and it was found that the inhibition of the expression of this gene precedes the onset of phenotypic differentiation by approximately 8 hr, is transient, and is accompanied by a brief retardation of cell proliferation, which resumes the normal rate within 24 hr of the exposure to the inducer.

HL 60 cells, isolated from a patient with a promyelocytic leukemia (1), have provided a useful model for maturation of leukemic cells. In culture, these cells show a low level of spontaneous differentiation, and essentially complete conversion to terminally differentiated forms can be induced by a variety of chemical compounds. For instance, dimethyl sulfoxide (2) and retinoic acid (3) produce conversion in 6–10 days to predominantly granulocytic cellular forms, while phorbol esters (4), lymphocyte-conditioned medium (5), and vitamin D derivatives (6–9) induce the monocytic/macrophage pathway of differentiation after 3–7 days in culture.

The long periods of time required for the phenotypic manifestations of the inducer action suggest that a complex series of steps is necessary for the expression of the differentiation program, and indicate to some that the process is stochastic in nature (10, 11). However, a relationship to a specific cell cycle event cannot be ruled out in experimental systems which utilize periods of induction greater than 24 hr, since this represents almost an entire generation cycle for optimally growing HL 60 cultures. In this situation cells are successively recruited into the differentiation program as they repeatedly traverse the cell cycle in the presence of the inducer, making it impossible to relate changes in cell cycle-linked events to the maturation process.

Several studies addressed the question of the minimal length of exposure of HL 60 cells to the inducer which can result in phenotypic expression of differentiation several days later. Treatment with dimethyl sulfoxide for 12 hr resulted in granulocytic differentiation of 20% of cells when examined 6 days later (10). Monocytic differentiation of HL 60 cells with 1,25-dihydroxycholecalciferol ($1,25(OH)_2D_3$), one of the most potent inducers of differentiation, appeared to require an even longer period of induction of differentiation, being variously estimated as 30 hr (11) or 18 hr (9), for a minimal expression of morphological or functional indicators of monocytic differentiation, as determined after a total of 4 (11) or 7 (9) days. These results suggested that, relative to events which occur at the molecular level,

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the presence of the inducer must be maintained in the culture medium for a very long period.

For these reasons we have optimized the conditions for exposure of HL 60 cells to $1,25(OH)_2D_3$ which permit more rapid differentiation of a significant proportion of these cells with clearly definable initiation of phenotypic differentiation. We have utilized this system to demonstrate that the induction of differentiation of HL 60 cells by a short exposure to $1,25(OH)_2D_3$ is preceded by a rapid but transient inhibition of the expression of c-myc oncogene, and by a transient inhibition of cell replication. These findings are consistent with a role for c-myc oncogene expression in the uncontrolled growth of HL 60 cells.

Materials and Methods. *Cell culture.* The HL 60 cells generously provided by Dr. Giovanni Rovera of the Wistar Institute, Philadelphia were subloned and continuously propagated in this laboratory for 12 months. The cells were incubated at 37°C in 25-cm^2 Falcon flasks using McCoys 5A modified medium (Flow Laboratories) supplemented with 1% glutamine (GIBCO) and 15% heat inactivated (56°C for 1 hr) fetal bovine serum (Hazelton Dutchland). To reduce the chance of an inapparent bacterial infection antibiotics were omitted from stock cultures, but 100 IU/ml penicillin–100 μg/ml streptomycin mixture (1% of the GIBCO stock solution) was present during the exposure to $1,25(OH)_2D_3$. Absence of mycoplasma infection was confirmed by the autoradiographic method (12).

Experimental cultures were initiated using cells resuspended in fresh culture medium at the density of 2×10^5 cells/ml. Cell proliferation was assessed by counting the cells in a Neubauer hemocytometer. Cell viability was determined by dye exclusion after exposure to 0.02% trypan blue, counting 300 cells. The doubling time of the viable cell population was found to be 24–36 hr.

Markers of monocytic differentiation. Cellular morphology was examined in air-dried smears stained with May–Grunwald–Giemsa solution. The principal cytological criterion of granulocytic differentiation was the presence of a nuclear constriction or segmentation, and monocytic differentiation was recognized by an oval or indented nucleus and a reduction in cell size from the undifferentiated blast form.

The occurrence of differentiation was confirmed by the following functional markers:

(a) Nitro blue tetrazolium (NBT) reduction. Approximately 2×10^6 cells were harvested and incubated at 37°C for 30 min in 1 ml of phosphate-buffered saline (PBS) 66 m*M* phosphate, 100 m*M* NaCl, pH 7.4, containing 0.1% NBT and 0.1 mg% phorbol-12-myristate-13-acetate in the presence of 1 m*M* *N*-ethylmaleimide (NEM). Smears were made by resuspending the cells in a drop of 0.5% albumin and spreading on a glass slide. Cells showing blue deposits of formazan were scored as positive for myelo-monocytic differentiation, counting a total of 300 cells on coded slides.

(b) Phagocytosis of opsonized sheep erythrocytes. Sheep red blood cells (rbc) (Hazelton Dutchland) were washed three times with PBS and placed on ice for 30 min with rabbit antisheep hemolysin diluted 1:500 with PBS. HL 60 cells and opsonized sheep rbc were mixed in 1:10 ratio and incubated at 37°C for 90–120 min. After the incubation period the adherent but not phagocytosed rbc were lysed by resuspending the cells in Tris-buffered (pH 7.2) 0.83% ammonium chloride for 2–5 min at room temperature. Air-dried smears were stained with May–Grunwald–Giemsa stain, and the proportion of cells containing ingested rbc enumerated counting 300 cells.

The monocytic nature of differentiation was assessed by the proportion of total cells in culture which were adherent to flask surfaces or to one another. Following this enumeration the adherent cells were detached by gentle pipetting and the cells were harvested by centrifugation at 1000 rpm (IEC 6000 centrifuge) for 10 min. Dried smears were fixed in an ice-cold mixture of 37% buffered Formalin (pH 6.6), acetone, and water (25: 45:30) at 4°C for 30 sec, and stained for nonspecific esterase activity by the method of Yam (13), using α-naphthyl butyrate (Sigma) in place of α-naphthyl acetate, and without using a counterstain. Three hundred cells were scored on coded slides.

Dot blot hybridization of RNA transcripts.

Total cellular RNA was extracted from 25 $\times 10^6$ cells for each time point by the guanidinium–hot phenol method (14) taking precautions to maintain sterile and RNase-free conditions, and stored in 70% ethanol at −70°C. For dot blotting the RNA was dissolved in sterile water, quantitated by absorbance at 260 nm, and blotted at several dilutions onto nitrocellulose filters which had been previously equilibrated with 20× SSC (1 × SSC is 0.15 *M* NaCl and 0.015 *M* sodium citrate). The blots were then air dried, baked overnight under vacuum at 80°C, and hybridized to cDNA nick-translated probes p myc 7.4 and p 72 (15, 16). p Myc 7.4 is a 1.2 kb cDNA fragment of the second and third exons of the c-myc gene inserted at the Pst 1 site of pBR322 (15). Probe p 72 is a cloned genomic DNA fragment from the human tumor cell line A 549 ligated to the pML-TK-Bgl 11 plasmid by Dr. Pater (16), and is known to contain an expressed portion of human tumor cell genome. Hybridization to either probe was performed according to Muller *et al.* (17). Briefly, the blots were incubated for at least 4 hr at 45°C with the prehybridization buffer (0.2 ml/cm^2) containing 0.75 *M* NaCl, 0.05 *M* sodium phosphate (pH 7.5), 0.005 *M* EDTA, 0.2% SDS, 10 mg/ml glycine, 5× Denhardt's reagent (1× is 0.02% each of Ficoll, bovine serum albumin, and polyvinylpyrrolidone), 0.25 mg/ml of denatured salmon sperm DNA, and an equal volume of deionized formamide. Subsequently, the blots were hybridized for 20 hr at 45°C with 1 × 10^6 cpm of nick-translated probe (sp act 10^7–10^8 cpm/μg DNA)/ml of hybridization buffer (0.05 ml/cm^2), which has the same composition as the prehybridization buffer except that the concentration of Denhardt's reagent was reduced to 1×. The blots were washed thoroughly three times (2 hr each wash) with 1× SSC at 50°C, air dried, and exposed to X-ray films with the use of an intensifying screen at −70°C for 72 hr. The intensity of developed autoradiograms was quantitated using a Hoefer Scientific Instruments (San Francisco, Calif.) G.S. 300 transmittance/reflectance scanning densitometer.

Chemicals. 1-α,25-Dihydroxycholecalciferol was provided by the courtesy of Dr. Gary Truitt and Dr. Milan Uskokovic, Hoffmann-La Roche, Nutley, New Jersey. The stock solution of this compound was prepared in absolute ethanol and stored at −20°C. Appropriate dilution with the culture medium was made just before use, and control medium was prepared by adding the equivalent volume of the ethanol solvent. This control medium had no distinguishable effect when compared with cells cultured in medium without the solvent.

Results. *1. The "pulse" procedure for induction of differentiation.* Exponentially growing HL 60 cultures of the strain cultivated in this laboratory show only a small proportion of spontaneously differentiated cells, generally not exceeding 3%. After exposure to $1,25(OH)_2D_3$ an increasing number of cells exhibit the characteristics of the more mature forms, as shown by the ability to phagocytose red blood cells, the nonspecific esterase reaction, and adherence to the culture flask wall (Table I). In our experiments 2.4 × 10^{-8} *M* $1,25(OH)_2D_3$ (10 ng/ml) produces 95% complete differentiation in approximately 70 hr. As reported by others (6, 9), increasing the concentration of this inducer accelerates the differentiation only slightly, and periods of exposure shorter than 16 hr are not sufficient for an effective induction of differentiation. However, when the short exposure to $1,25(OH)_2D_3$ takes place in medium not supplemented with fetal calf serum, a significant proportion of cells acquires the differentiated phenotype when examined after a lapse of time approximately equivalent to a cell cycle generation time (Table I).

These experiments also indicated that a 4-hr period of induction is optimal for the appearance of a discrete cohort of differentiated cells. Shorter periods of exposure to $1,25(OH)_2D_3$ resulted in smaller and less reproducible numbers of differentiated cells, while periods longer than 4 hr did not markedly augment differentiation. It was also felt that the period of incubation without serum should be kept as short as possible, to minimize a chance of reduced cell viability due to the lack of essential nutrients. The 4-hr period without serum did not produce detectable cytotoxicity or loss of viability (data not included).

The induction period was followed by a thorough washing of the cells to remove

TABLE I. COMPARISON OF SEVERAL DIFFERENTIATION PARAMETERS OF HL 60 CELLS EXPOSED TO 10^{-7} *M* $1,25(OH)_2D_3$

Time in hr		Cell morphology					
Period of exposure to inducer	Examined at	Blast forms (%)	Granulocytic forms (%)	Monocytes and precursors (%)	Phagocytic cells (%)	NSE reaction (%)	Cell adherence
0	0	99 ± 3	<1	<1	<1	<1	0
4	4	99 ± 2	<1	<1	<1	<1	0
4	8	98 ± 5	<1	1 ± 2	<1	2 ± 2	0
4	12	91 ± 5	<1	9 ± 3	8 ± 6	12 ± 3	0
4	24	72 ± 9	4 ± 3	24 ± 6	28 ± 2	34 ± 3	**
4	48	88 ± 12	<1	12 ± 5	16 ± 1	15 ± 9	*
4	72	94 ± 6	<1	6 ± 3	6 ± 4	4 ± 4	0
24	24	27 ± 14	7 ± 4	66 ± 10	34 ± 3	72 ± 9	***

Note. The values shown for percentages represent the mean of three experiments ± SD. Cell adherence was assessed by the proportion of cells forming clumps and adhering to culture flask wall, ***corresponding to 50–75% adherent cells. The 4-hr exposure to $1,25(OH)_2D_3$ took place in the absence of serum.

$1,25(OH)_2D_3$ from the medium. Nonetheless, in early experiments the sharp rise in the proportion of differentiated cells which began at approximately 10 hr from induction and peaked between 30–40 hr (see below) was followed by a more gradual increase in differentiated cells which continued during the period of observation. However, when the washing of HL 60 cells was repeated 2 hr after the induction period, and the cells incubated in another batch of fresh medium, this increase did not continue after 36 hr. This indicates that intracellular $1,25(OH)_2D_3$ diffuses out of the cells when its external concentration is lowered, and concentrations effective for induction may be removed by a repeat wash after several hours. Under these conditions a cohort of HL 60 cells with the differentiated phenotype is observed (Fig. 1).

2. Chronology of cohort differentiation of HL 60 cells. There is no phenotypic evidence of differentiation of HL 60 cells during the

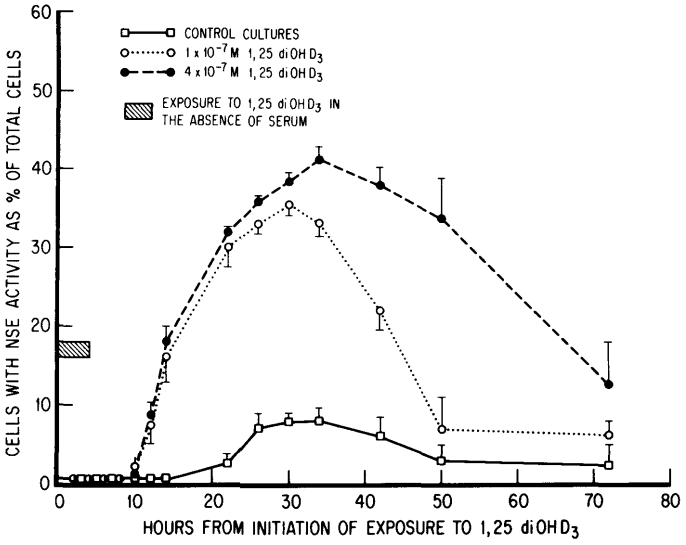

FIG. 1. Kinetics of differentiation of HL 60 cells induced by the pulse method. The symbols represent the mean values of four experiments ± SE.

4-hr exposure to $1,25(OH)_2D_3$ or for approximately 6 hr afterward. After this latent period several indicators of monocytic differentiation become apparent and peak at 28–32 hr, depending on the concentration of $1,25(OH)_2D_3$ used (Table 1 and Fig. 1). A fourfold increase in the concentration of this inducer produced only a small (approximately 5%) increase in the height of this peak, but the rate of decrease in the proportion of differentiated cells is slower after induction with the higher concentration of $1,25(OH)_2D_3$.

Control cultures not exposed to $1,25(OH)_2D_3$ but handled in an identical manner to the experimental groups, including a 4-hr period in the absence of serum, showed a small but reproducible peak of differentiated cells at approximately the same time as the vitamin-treated cultures (Fig. 1). Thus, a small proportion of phenotypically differentiated cells observed at 25–42 hr may have been induced by the handling rather than by the inducer.

The 4-hr pulse of $1,25(OH)_2D_3$ produced a small but reproducible and statistically significant retardation in the rate of cell proliferation, determined by cell numbers per milliliter of culture, 24 hr after induction (Fig. 2). Thereafter, the cultures showed identical rates of growth, indicating that the retardation occurred only within the first 24 hr of exposure to the inducer. The two concentrations of $1,25(OH)_2D_3$ used in these experiments produced similar degrees of growth retardation (Fig. 2). Thus, if the inducing drug is removed, HL 60 cells can undergo at least 3 divisions at approximately normal rate although a large proportion of the cells in culture are differentiating.

3. Gene expression during cohort differentiation of HL 60 cells. It has been reported that the expression of c-myc oncogene is reduced in HL 60 cells induced to differentiate by an exposure to $1,25(OH)_2D_3$ (7), raising the possibility that these events are causally related. It therefore appeared important to determine if significant changes in c-myc expression occur when the pulse procedure induction described here is employed, and the chronology of the genomic and phenotypic changes. We observed that, assuming a steady state of c-myc mRNA metabolism, the expression of this gene is changed little during the first 2 hr of exposure to 2.4×10^{-8} *M* $1,25(OH)_2D_3$, but falls sharply thereafter, being less than 20% of the basal level at 4 hr, the end of the induction period (Fig. 3,

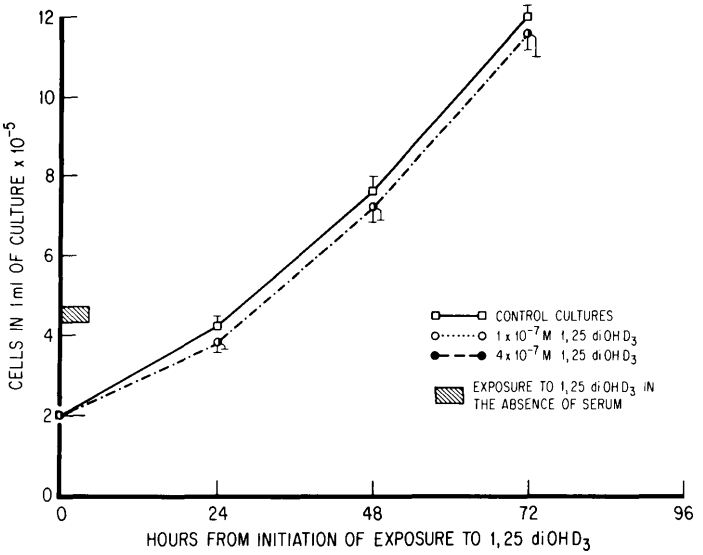

FIG. 2. Rate of growth of HL 60 cultures induced to differentiate by the pulse method. The symbols represent the mean values for four experiments ± SE. Student's *t* test with paired values showed that the means of treated cultures at 24 hr were significantly different from the control at $P < 0.05$; at 48 and 72 hr the values for *P* were >0.05.

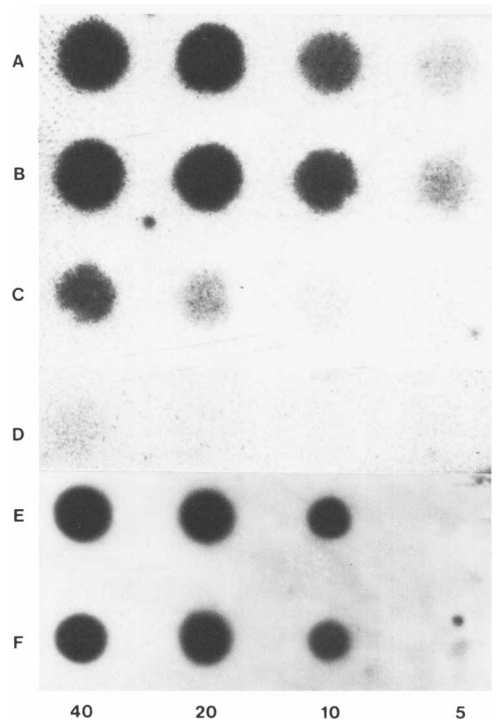

FIG. 3. (Rows A–D) Autoradiograms of dot blots of total RNA extracted from HL 60 cells immediately following treatment with 2.4×10^{-8} *M* $1,25(OH)_2D_3$ for varying periods of time. The RNA was applied to nitrocellulose filters and hybridized to c-myc cDNA nick-translated with ^{32}P. Note a reduction in cellular content of c-myc mRNA after periods of exposure to $1,25(OH)_2D_3$ longer than 2 hr. (Row A) Serial dilutions (indicated as μg) of RNA from untreated HL 60 cells. (Row B) RNA from HL 60 cells exposed to 2.4×10^{-8} *M* $1,25(OH)_2D_3$ for 2 hr. (Row C) Cells exposed to the inducer for 4 hr. (Row D) Cells exposed to the inducer for 6 hr. (Rows E and F) Dot blots hybridized to a cDNA probe of gene p 72. There is no evidence of any change in the expression of this gene during 6 hr of exposure of HL 60 cells to 2.4×10^{-8} *M* $1,25(OH)_2D_3$. (Row E) Serial dilutions of RNA from untreated cultures. (Row F) Serial dilutions of RNA from cultures exposed to 2.4×10^{-8} *M* $1,25(OH)_2D_3$ for 6 hr. Concentrations of RNA indicated along the bottom line are in μg of RNA applied to the filter.

rows A–C). If the exposure to $1,25(OH)_2D_3$ is continued, c-myc mRNA becomes barely detectable (<2% of control) after 2 additional hr (Fig. 3, row D). This effect is considerably more marked than that described by Reitsma *et al.* (7), who found 50% reduction in c-myc RNA levels at 4 hr of treatment, but still detected significant levels of c-myc mRNA in HL 60 cells treated for 72 hr with 10^{-7} *M* $1,25(OH)_2D_3$. The shut-down of c-myc gene is at least to some extent specific, since the expression of an unrelated gene, p 72 (16), is unaltered by the treatment of HL 60 cells with $1,25(OH)_2D_3$ (Fig. 3, rows E and F).

These experiments were repeated using the system of induction described here with similar results. It was found that serum withdrawal had no detectable effect on c-myc RNA levels in control cultures, but the presence of 1×10^{-7} *M* $1,25(OH)_2D_3$ resulted in a dramatic decrease in c-myc RNA, maximal at 6 hr (4 hr with the inducer plus 2 hr in normal medium). At this time c-myc RNA concentration was less than 2% of the levels found in control cultures subjected to a similar regimen of serum deprivation (Fig. 4, rows C and F). Interestingly, c-myc expression appears to recover rapidly, reaching 75% of control value at 10 hr, and approximately normal values are observed at 14 hr (Fig. 4, rows D and E).

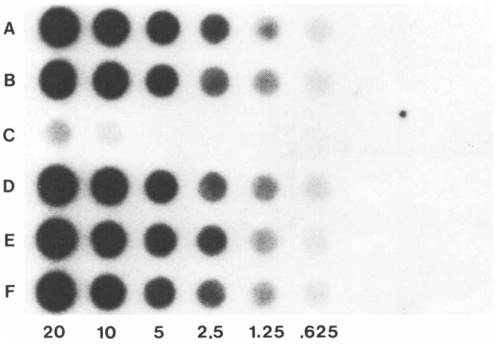

FIG. 4. Autoradiogram of dot blots of RNA extracted from HL 60 cells at 2 hr and then at four-hourly intervals of the standard induction system. The 4-hr period of exposure to 1×10^{-7} *M* $1,25(OH)_2D_3$ in the absence of serum was followed by reincubation in normal medium, with thorough washes at the end of the treatment, and again 2 hr later. The RNA on nitrocellulose filters was hybridized to c-myc cDNA nick-translated with ^{32}P. There is a marked drop in the levels of c-myc RNA at 6 hr of this treatment. (Row A) Serial dilutions (indicated as μg of RNA along the bottom of the autoradiogram) of RNA extracted from untreated cultures at time 0. (Row B) RNA from cultures exposed to 1×10^{-7} *M* $1,25(OH)_2D_3$ for 2 hr. (Row C) RNA from treated cultures at 6 hr. (Row D) RNA from treated cultures at 10 hr. (Row E) RNA from treated cultures at 14 hr. (Row F) RNA from untreated cultures, but incubated for 4 hr without serum, at 6 hr.

The finding that the level of c-myc mRNA is reduced during the initiation of differentiation by the "vitamin D pulse" procedure is consistent with the view that a decrease in the expression of c-myc gene acts as a stimulus for the events which lead to terminal differentiation of HL 60 cells.

Discussion. The induction of monocytic differentiation of HL 60 cells by a brief exposure to a vitamin D analog should provide a system useful for the study of several puzzling aspects of the control of differentiation of leukemic cells. It may permit a clearer picture of the relationship of the events which initiate a differentiation pathway to the phases of the cell cycle, the importance of which is suggested by the transient prolongation of the generation time observed during induction of differentiation by the procedure described here (Fig. 2), and in several other differentiating cell populations (18–22). Further, the well-delineated progression toward the differentiated phenotype (Fig. 1) may facilitate a step by step analysis of the biochemical events which lead from the initiation of the process, presumably at genomic level, to its final expression as changes in cell structure, enzyme levels, and membrane properties, the well-documented markers of monocyte-like differentiation (6–9, 13).

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