Macromolecular and Cell Cycle Effects of Different Classes of Agents Inducing the Maturation of Human Myeloblastic Leukemia (ML-1) Cells

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ABSTRACT

The effect of various classes of differentiation-inducing agents on macromolecular synthesis was studied in a human myeloblastic leukemia cell line (ML-1). Antineoplastic drugs such as 1β-d-arabinofuranosylcytosine, daunorubicin, and actinomycin D caused early inhibition of DNA synthesis, which generally preceded the accrual of differentiation markers. In contrast, retinoic acid and conditioned medium from mitogen-stimulated leukocytes caused a delayed decline in DNA synthesis, which accompanied the appearance of maturing morphology. With 12-O-tetradecanoylphorbol-13-acetate, the decline in DNA synthesis was temporally linked to the onset of maturation, and this agent evidenced some properties of both the antineoplastic agents and the more physiological inducers, retinoic acid and conditioned medium. Antineoplastic agents and conditioned medium, when applied simultaneously, induced differentiation in an additive or synergistic manner, simulating the effects of 12-O-tetradecanoylphorbol-13-acetate.

RNA and protein synthesis continued during maturation induced with all these agents, although a partial reduction in RNA synthesis was observed at later time points (24 hr). Agents incapable of inducing differentiation, such as cordycepin and cycloheximide, were characterized by a lack of sustained inhibition of DNA synthesis and/or by early (3 hr) inhibition of RNA or protein synthesis.

The decline in DNA synthesis caused by the inducing agents was accompanied by decreased cell cycle progression, cells accumulating largely in G1 phase. With daunorubicin and actinomycin D, block of the G1-S transition was evident at 24 hr, whereas with conditioned medium and retinoic acid, accumulation in G1 occurred in a progressive fashion, >77% of cells residing in this phase on Day 6. Maximal inducing doses of 12-O-tetradecanoylphorbol-13-acetate (>80% differentiation) caused an accumulation of cells in G1, as well as an accumulation of cells with a G2-M-phase DNA content (approximately 40%).

These observations indicate that early inhibition of DNA synthesis, with sparing of RNA and protein synthesis, is characteristic of the differentiation-inducing antineoplastic drugs examined. These agents may induce differentiation by inhibition of the proliferation path, whereas conditioned medium and retinoic acid may act by the stimulation of differentiation paths. Differentiation can be enhanced by the simultaneous application of agents targeting both of these paths.

INTRODUCTION

A variety of agents including polar compounds (7, 16, 24, 42), phorbol esters (24, 32, 35, 42), anticancer drugs (3, 11, 15, 34, 44, 45, 48), retinoic acid (4, 23, 41), and conditioned medium factors (21, 43, 54) are capable of stimulating the differentiation of leukemic cells and other neoplastic cell types. However, the mechanisms of differentiation-induction by these diverse substances are not well understood.

Various mechanisms have been proposed to be involved in the maturation-inducing actions of these agents. Demonstrated biochemical features of several inducers include inhibition of DNA synthesis and function (11, 45), alteration of chromatin structure (16, 49), modification of DNA methylation (5, 25), and effects on the RNA:protein ratio (19). Actions of inducing agents at the cell surface (8, 49, 53), alteration of the program of RNA transcription (29), modulation of ion-regulating ATPases (16, 39), and inhibition of phospholipid synthesis (20) are among the changes that have been considered to participate in differentiation induction.

Past reports have disagreed on the role that DNA synthesis plays in differentiation induction. In some systems (22, 30, 38, 40), DNA synthesis is deemed essential for maturation; in others (12, 13, 27, 31, 33, 50, 51), it is not considered to be required, and, in some neoplastic cells, inhibition of DNA synthesis is held to contribute to the differentiation process (1, 2, 44, 45).

In conjunction with studies on the differentiation response of a human myeloblastic leukemia (ML-1) cell line (42–46), we have evaluated the effects of several physiological and nonphysiological differentiation-inducing agents, focusing on DNA, RNA, and protein synthesis, and on cell cycle progression. These studies were based upon the hypothesis that inhibition of DNA synthesis may promote the differentiation of ML-1 cells and that maintenance of RNA and protein synthesis may be required for their maturation. A preliminary report of these studies has been presented (9).

MATERIALS AND METHODS

Materials

The ML-1 cell line was generously provided by Dr. J. Minowada, Hines Veterans Administration Medical Center, Hines, IL. RPMI 1640 medium and FBS4 were purchased from Grand Island Biological Co., Grand Island, NY. Tissue culture flasks were obtained from Corning Glass Works, Corning, NY, or from Costar, Cambridge, MA.

ara-C was purchased from The Upjohn Co., Kalamazoo, MI. Dauno-

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Received November 23, 1983; accepted March 8, 1984.

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mycin HCl, actinomycin D, retinoic acid (all-trans), cordycepin, cycloheximide, puromycin dihydrochloride, vincristine sulfate, penicillin G, streptomycin sulfate, and pokeweed mitogen were supplied by Sigma Chemical Co., St. Louis, MO. TPA was obtained from Chemicals for Cancer Research, Inc., Eden Prairie, MN, and was stored in DMSO as described previously (42). The final concentration of DMSO (<0.003%) was without effect on the cells. The remaining drugs were dissolved in PBS or absolute ethanol, the final concentration of either solvent being without measurable effects on the cells.

Buffcoats from the blood of healthy human volunteers were furnished by the Roswell Park Memorial Institute Blood Bank, and conditioned medium from the pokeweed mitogen-stimulated mononuclear cells was prepared in our laboratory as described in a prior report (43). Ficoll-Hypaque was purchased from Pharmacia Fine Chemicals, Piscataway, N.J. Bovine erythrocytes were supplied by the Springfield Laboratories of Roswell Park Memorial Institute, Springville, NY, and rabbit antitibovine erythrocyte antibodies were prepared by Dr. J. Minowada.

Sterile aqueous solutions of [methyl-3H]dThd (hereafter called [3H]-dThd; 30 to 38 Ci/mmol), [5-3H]uridine (hereafter called [3H]uridine; 14 Ci/mmol), and [4,5-3H]leucine (hereafter called [3H]leucine; 55 to 58 Ci/mmol) were purchased from ICN Pharmaceuticals, Inc., Chemical and Radioisotopes Division, Irvine, CA, with radioisotopic purity being monitored periodically by thin-layer chromatography. GF/C glass microfiber discs (2.4 cm) were manufactured by Whatman Chemical Separation, Ltd., Maidstone, Kent, England.

Methods

Cell Culture. ML-1 cells were maintained in suspension culture in RPMI 1640 medium supplemented with 7.5% heat-inactivated FBS, essentially as described previously (42). Mycoplasma-free cells were stored (10% DMSO) in liquid nitrogen, and aliquots were thawed at intervals of 2 to 3 months, washed twice, and allowed to resume logarithmic growth prior to use in the assays described below.

For in vitro testing of differentiation-inducing agents, cells were harvested from logarithmically growing cultures and resuspended at a concentration of 3 x 10⁶ viable cells/ml in RPMI 1640 containing 10% FBS, 100 units penicillin/ml, 100 µg streptomycin/ml, and the desired drug concentration. In experiments of longer than 3 days duration, sufficient drug-containing medium was added on Day 3 to readjust the cell number to 3 x 10⁶ viable cells/ml.

In examining the biological and biochemical effects of antineoplastic agents, TPA, retinoic acid, and conditioned medium from mitogen-stimulated human leukocytes, drug concentrations which produced maximal conversion to maturing phenotype were selected based upon preliminary dose-response studies (data not shown). Drugs without differentiation-inducing properties were studied at concentrations that produced a reduction of proliferation similar to that seen in differentiating cultures.

Assay of Cell Growth and of Differentiation-associated Characteristics. Cell growth was assayed by hemocytometer, and viability was estimated by trypan blue dye exclusion. By this criterion, control cultures were found routinely to contain ≥92% viable cells.

Assay of the appearance of F₄₅ was performed using standard techniques for erythrocyte-antibody rosette formation, as described previously (42). In brief, cells were washed and mixed with 100 µl of PBS and 50 µl of a suspension of bovine erythrocytes coated with rabbit anti-bovine erythrocyte antibodies. Cells were incubated for 1 hr at room temperature after centrifugation at 200 x g for 2 min. Viable mononuclear cells with at least 3 attached erythrocytes were scored as F₄₅ positive, and at least 100 viable cells were scored for each determination. For the preparation of antibody-coated erythrocytes, a 5% solution of PBS-washed bovine erythrocytes was mixed 1:1 with rabbit anti-bovine erythrocyte antibodies (diluted 1:40 with PBS except for assay of conditioned medium-treated cells where the dilution was 1:160), incubated at 37°C for 30 min, then washed, and resuspended in PBS at a concentration of 0.5%.

The appearance of morphological differentiation was assessed using stained slide preparations (42). Cells were mixed with 0.1 ml 7.5% bovine albumin Fraction V and 0.4 ml PBS, and slides were prepared by centrifugation in a Shandon Cytospin II. The slides were fixed in methanol and stained with Wright's stain for 3 min and Giemsa stain for 6 min. Monocytic differentiation was scored using established criteria (28): macrophage-like cells manifesting a reniform nucleus and a decreased nucleocytoplasmic ratio; monocyte-like cells exhibiting a more centrally located maturing nucleus and less cytoplasmic enlargement than macrophage-like cells; and intermediate-stage (promonocyte-like) cells typically displaying an immature nucleus in the presence of moderate cytoplasmic enlargement. Cells induced to differentiate along the granulocytic series were scored as myeloblasts, promyelocytes, myelocytes, metamyelocytes, band forms, or segmented forms, in accordance with standard criteria (28).

NBT dye-reducing ability was measured as described previously (42). Viable cells containing blue-black formazan deposits were scored as NBT positive, with at least 100 viable cells being counted for each experimental point.

Assay of acid phosphatase activity was performed by measuring the enzymatic hydrolysis of p-nitrophenyl phosphate, as detailed elsewhere (35). Briefly, lysates (about 5 x 10⁶ viable cells/40 µl) prepared by sonication in distilled water were incubated for 15 min at 37°C in the presence of 0.4 ml of 38 mM sodium citrate, pH 4.8, containing 0.1% Triton X-100 and 4.2 mM p-nitrophenyl phosphate. The reaction was stopped by the addition of 0.8 ml of 0.1 M NaOH, and the concentration of p-nitrophenol was determined spectrophotometrically at a wavelength of 410 nm.

Cell adherence was quantitated by comparing the number of cells in suspension with the number of cells obtained by harvest of the twiced-washed monolayer, using established methods for collection of adherent cells (37).

Assay of the Incorporation of Radiolabeled Precursors Into Acid-insoluble Material. Assessment of DNA, RNA, and protein synthesis was performed using standard procedures (36). Briefly, ML-1 cells were pulse labeled for 3 hr with either [3H]dThd (0.032 µCi/ml final concentration), [3H]uridine (0.032 µCi/ml), or [3H]leucine (0.4 µCi/ml). Cells were washed with PBS and extracted with 5% trichloroacetic acid (106 viable cell equivalents/ml) for 20 min on ice. One-ml aliquots were placed on glass microfiber filter discs under moderate suction, and the discs were washed 4 times with ice-cold 5% trichloroacetic acid, once with absolute ethanol, and twice with acetone. After drying the discs at 50°C, radioactivity was quantitated by liquid scintillation counting. This procedure was found to remove ≥98% of the unincorporated radioisotopes, and quenching of the disc was found to be minimal (usually about 10%). Where adherence of cells to the tissue culture vessel was encountered, adherent cells were harvested using published methods (37). The removal of nonviable cells, where present, was not found to substantially affect the results.

Low concentrations of radioisotopic precursors were used in these assays because ML-1 cells were found to be sensitive to [3H]dThd and [3H]uridine, 50% inhibition of cell growth being encountered at concentrations in the range of 0.10 to 0.15 µCi/ml. At these concentrations, approximately 25% cell kill was seen, and this effect was not prevented significantly by the addition of nonradioactive nucleosides at non-growth-inhibitory concentrations. These events are similar to the "tritiated thymidine suicide" reported in other cycling hematopoietic stem cells (10). The low concentrations of radioisotopic compounds used in our experiments were found to have minimal cytostatic effects, while providing linear precursor incorporation.

Assay of Cell Cycle Distribution. Cell cycle distribution was determined by flow cytometry, after staining nonfixed cells with the DNA-specific fluorochrome 4',6-diamidino-2-phenylindole in the presence of 0.2% Triton X-100 (47). Fluorescence intensity was measured on an ICP-22 flow cytometer using UV excitation (peak transmission at 360 nm) with an emission filter of 450 to 490 nm. A total of 30,000 to 50,000
cells was counted for each histogram. Where indicated, viable cells were separated by Ficol-Hypaque gradient centrifugation (42) before staining. For disaggregation of TPA-treated cells, washed cells were agitated (37°) in 0.02% EDTA for 5 to 15 min. DNA content distributions were analyzed with an Apple II microcomputer (18), using an algorithm which fits the G, and G2-M peaks to gaussian curves and which, for S phase, constructs a rectangular box with a height equal to the average height of mid-S phase and a length equal to the distance between the G, and G2-M peaks.6

RESULTS

Appearance of FcR-bearing Cells after Treatment with Differentiation-inducing Agents. The time course of emergence of FcRs was monitored to determine the utility of this parameter for the early detection of maturation induction. As shown in Chart 1A, the FcR marker began to increase on about Day 2 in cultures treated with ara-C, daunorubicin, or actinomycin D. FcR-bearing cells continued to accrue after this time, approaching maximal levels on Day 6. A marked increase in the FCR index was observed as early as Day 1 in cultures treated with conditioned medium and within 2 to 3 days after administration of the antineoplastic agents.

Effects of Antineoplastic Differentiation-inducing Agents on [3H]dThd, [3H]uridine, and [3H]leucine Incorporation. As shown in Chart 2, ara-C, daunorubicin, and actinomycin D caused extensive inhibition of [3H]dThd incorporation, while having less pronounced effects on [3H]uridine and [3H]leucine incorporation. In cells exposed to ara-C, incorporation of [3H]dThd into acid-insoluble material was inhibited by about 90% within 3 hr after drug addition (Chart 2, IA). [3H]dThd incorporation declined to nearly undetectable levels (5% of control) on Day 3, and this reduction was maintained through Day 6, in agreement with the hypothesis that interference with DNA synthesis may contribute to the differentiation-induction activity of this agent.

Inhibition of DNA synthesis preceded the accrual of significant numbers of FcR-bearing cells (Chart 1A) and thus appeared to be an early event in the induction process. DNA synthesis inhibition also occurred prior to the increase in acid phosphatase activity (Chart 2, IC), which began on about Day 3, as morphological differentiation along the monocytic pathway was progressing. In contrast to the marked and sustained decline in [3H]dThd incorporation, [3H]uridine and [3H]leucine incorporation were maintained above 50% of control throughout differentiation induced with ara-C (Chart 2, IB), in accordance with the hypothesis that continued RNA and protein synthesis may be required for the maturation process.

In daunorubicin-treated cultures, [3H]dThd incorporation fell to <2% of the control value within 24 hr (Chart 2, IIA), largely preceding the increases in the FcR index (Chart 1) and in acid phosphatase activity (Chart 2, IIC). Unlike [3H]dThd incorporation, [3H]uridine incorporation (Chart 2, IIB) continued throughout the induction period, albeit at reduced levels (50 to 78% of control on Days 1 to 6). In spite of the partial decline in [3H]uridine incorporation, [3H]leucine incorporation, although somewhat reduced on Days 1 and 2, was restored during the later stages of maturation (Days 3 to 6) to 65% of the control level.

Actinomycin D decreased [3H]dThd incorporation by approximately 65% on Day 1, inhibition being essentially complete by Day 2 (Chart 2, IIIA). [3H]uridine incorporation (Chart 2, IIIIB) was also affected by actinomycin D. Little decrease (11%) in [3H]uridine incorporation was seen at 3 hr, with a 50% reduction...
being observed at 24 hr. [3H]Uridine incorporation remained at about 31% of the control level at 48 hr, when [3H]dThd incorporation had declined to about 5% of control. Low levels of RNA synthesis, amounting to approximately 18% of control on Days 3 to 6, persisted during the remainder of the induction period. [3H]Leucine incorporation also underwent a reduction (57%) up to Day 3, after which time a return to near control levels was seen.

These observations indicate that the biochemical properties of these antineoplastic differentiation-inducing agents include extensive inhibition of DNA synthesis in the presence of some continued RNA and protein synthesis.

Effects of Agents without Differentiation-inducing Activity on [3H]dThd, [3H]Uridine, and [3H]Leucine Incorporation. Cordycepin, cycloheximide, puromycin, and vincristine have been shown previously to have little differentiation-inducing activity (44, 45). As shown in Table 1, these agents were characterized by a lack of sustained inhibition of DNA synthesis and/or by early inhibition of either RNA or protein synthesis. For example, cordycepin caused only transient inhibition of [3H]dThd incorporation (at 24 hr), while producing considerable (60%) inhibition of [3H]Uridine incorporation as early as 3 hr after drug addition. Cycloheximide inhibited [3H]dThd incorporation but to 48 hr but also caused rapid, extensive, and sustained inhibition of [3H]Leucine incorporation. Neither puromycin nor vincristine caused sustained inhibition of DNA synthesis, a principle effect of these agents being the slowly developing inhibition of [3H]Leucine incorporation.

**Effects of Antineoplastic Differentiation-inducing Agents on Cell Cycle Distribution.** The ML-1 cell line used in this investigation contained 2 aneuploid cell subpopulations (Chart 3), a minor population with a DNA ploidy index of 1.1 (approximately 25% of the total population or less) and a predominant hypertetraploid population with a DNA index of 2.2. The cell cycle effects of various agents on these 2 subpopulations were similar and were evaluated on the basis of changes in the DNA distribution of the predominating tetraploid population.

In the presence of ara-C, an accumulation of cells in S phase was seen on Day 1 (Chart 3; Table 2), reflecting delayed progression through this phase. Between Days 1 and 5, the proportion of cells in S phase fell to below the control value, as is evident from the reduced proportion of cells in G, of the diploid subpopulation.

**Effects of Anticancer Agents on Cell Cycle Distribution.** The ML-1 cell line used in this investigation contained 2 aneuploid cell subpopulations (Chart 3), a minor population with a DNA ploidy index of 1.1 (approximately 25% of the total population or less) and a predominant hypertetraploid population with a DNA index of 2.2. The cell cycle effects of various agents on these 2 subpopulations were similar and were evaluated on the basis of changes in the DNA distribution of the predominating tetraploid population.

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decreased at this time point, in accord with the observed decline in [3H]dThd incorporation (Chart 2). Daunorubicin additionally induced some G2-M block, which was evident in the tetraploid population at early time points. The presence or absence of G2-M block in the diploid population could not be established because of the large number of G1-phase tetraploid cells with an equivalent DNA content. On Day 6, cells treated with daunorubicin or actinomycin D resided largely in the G1 compartment. In contrast to these agents, cycloheximide did not cause the population of S-phase cells to decline on Day 1, apparently slowing progression through all cell cycle phases.

Chart 5. Time-dependent effects of conditioned medium and retinoic acid on [3H]dThd, [3H]uridine ([3H]Urd), and [3H]leucine ([3H]Leu) incorporation in relation to ML-1 cell differentiation. ML-1 cells were incubated in the presence or absence of 20% conditioned medium (Column I) or 5 × 10⁻⁸ M retinoic acid (Column II) and assayed at the indicated time points for incorporation of [3H]dThd (A), [3H]uridine and [3H]leucine (B), and for maturing cells (C). A, incorporation of [3H]dThd into acid-insoluble material in control and treated samples. B, incorporation of [3H]uridine and [3H]leucine into acid-insoluble material as a percentage of control. The first time point shown is 3 hr. C, maturing cells of the monocytic series (Column I) as a percentage of the viable cell population (I, III, and □), intermediate, monocyte-like, and macrophage-like cells, respectively, or maturing cells of the granulocytic series (Column II), with I, II, and □ representing myelocytes, myelocytes, and band-segmented forms, respectively. The mean of concurrent controls, which did not change significantly during the 6-day incubation period, is shown at the origin. Segmented forms accounted for <2% of the retinoic acid-treated cell population on Day 6. Points, mean of 2 experiments, with duplicate samples in each experiment; bars, S.E., with the bars in C referring to the total maturing cell population.

Effects of Conditioned Medium and Retinoic Acid on [3H]-dThd, [3H]-Uridine, and [3H]-Leucine Incorporation and on Cell Cycle Distribution. Conditioned medium from mitogen-stimulated human leukocytes has been shown previously to contain a protein factor which induces ML-1 cells to differentiate to monocytic- and macrophage-like forms (43, 46), and retinoic acid stimulates these cells to differentiate along the granulocytic pathway. As shown, differentiation induced with conditioned medium or retinoic acid (Chart 5, I and II) was not preceded by a sharp decline in DNA synthesis (Chart 5, I and III), in contrast to the pattern seen with the antineoplastic inducers. Instead, some [3H]dThd incorporation continued during the first 3 days of differentiation induction. With conditioned medium, a progressive decline in incorporation (to 65% of the initial value) was observed through Day 3. A further decline in DNA synthesis was seen on Day 6, at which time 80% of cells manifested maturing phenotype. Accrual of FcR-bearing cells occurred within 1 day of
exposure (Chart 1B), demonstrating that the appearance of this index need not be preceded by the cessation of DNA synthesis.

At early time points, [3H]dThd incorporation in cultures treated with retinoic acid paralleled that in controls (Chart 5, IIA), a decrease becoming apparent after Day 3. Morphological differentiation in these samples was characterized by the transition of cells from the myeloblast-promyelocyte phenotype to myelocyte, metamyelocyte, and band forms (Chart 5, IIC). Some maturation was seen on Day 2, maximal differentiation becoming evident on about Days 5 and 6. Thus, the decline in DNA synthesis in retinoic acid-treated cultures accompanied the appearance of large numbers of maturing cells.

Conditioned medium and retinoic acid had only moderate effects on RNA and protein synthesis (Chart 5, B), although a considerable decline was seen on Day 6 after retinoic acid treatment. Since drug effects on pool sizes of dThd, uridine, and leucine were not measured, assessment of the incorporation of radioactive precursors represents a qualitative estimate of the presence or absence of continued macromolecular synthesis. Using this measure, conditioned medium and retinoic acid clearly differ from the antineoplastic agents with respect to their effects on DNA synthesis, but they appear broadly similar in their effects on RNA and protein synthesis.

The continued DNA synthesis in conditioned medium- and retinoic acid-treated cultures at early time points was reflected in the DNA content distribution histograms, as shown in Chart 4B. With conditioned medium, 34% of cells remained in S phase on Day 1 (Table 2), as compared to 46% in controls, and with retinoic acid, no decline in the S-phase population was seen at this time. A loss of S-phase cells occurred at later time points, and, after 6 days, cells were found largely in the G1 compartment. Thus, the initial cell cycle effects of these agents contrasted with those of daunorubicin or actinomycin D; however, after prolonged exposure, the cell population resided largely in G1, in all cases.

Effects of TPA on [3H]dThd, [3H]Uridine, and [3H]Leucine Incorporation. As shown in Chart 6, TPA-induced ML-1 cell maturation was accompanied by dose-dependent inhibition of [3H]dThd incorporation. A concentration of 0.42 × 10^{-10} M TPA, which produced little inhibition of [3H]dThd incorporation (Chart 6, IA), had little effect on morphological differentiation (Chart 6, IIC). A concentration of 0.83 × 10^{-10} M TPA caused partial inhibition of [3H]dThd incorporation (79% on Day 1) and maturation in a fraction of the cell population (31% on Day 3). Doses of TPA which caused maximal inhibition of [3H]dThd incorporation caused >80% conversion to maturing phenotype.

The effects of TPA on RNA and protein synthesis (Chart 6, IIB) were less pronounced than its effect on DNA synthesis and were not closely correlated with differentiation induction. At a concentration of 0.83 × 10^{-10} M TPA, [3H]uridine incorporation was decreased by 54%, as compared to a 79% decrease in [3H]dThd incorporation. Higher concentrations caused only minimal further decreases in [3H]uridine incorporation, which remained at 36 to 50% of control at concentrations producing >94% inhibition of [3H]dThd incorporation. [3H]Leucine incorporation was maintained at greater than 83% of control with all doses of TPA and was increased to above control levels at high drug concentrations.

A kinetic analysis of the effects of 1.7 and 5.0 × 10^{-10} M TPA is shown (Chart 6, IIB). At the maximally effective concentration of 5 × 10^{-10} M TPA, inhibition of DNA synthesis was rapid and sustained (Chart 6, IIA). [3H]dThd incorporation began to fall after 9 hr, reaching undetectable levels at 24 hr. Cessation of DNA synthesis appeared to be temporally linked to the onset of differentiation, some maturing cells beginning to emerge at 24 hr (Chart 6, IIC). RNA synthesis declined at a slower rate (Chart 6, IIB), a progressive reduction in [3H]uridine incorporation (to 18% of control at 72 hr) occurring during the accrual of maturing cells. [3H]Leucine incorporation was maintained at ≥85% of control throughout differentiation.

The effects of 1.7 × 10^{-10} M TPA on DNA synthesis (Chart 6, IIA) were less pronounced than those obtained with the 5.0 × 10^{-10} M concentration (Chart 6, IIA). A residual level of [3H]dThd incorporation was maintained at 24 hr and beyond, and RNA and protein synthesis were maintained at >49% of the control level during the entire maturation period (Chart 6, IIB).

Effects of TPA on Cell Cycle Distribution. TPA was found capable of affecting cell cycle progression in 2 ways. At all effective concentrations, a G2-M block was established, and, in the higher concentration range, a G2-M block also occurred. The G2-M block was dose dependent, a concentration of 0.83 × 10^{-10} M TPA causing a small decline in the S-phase compartment (Chart 7; Table 2) and the appearance of some maturing cells (Chart 6). Concentrations above 3.3 × 10^{-10} M caused maximal G2-M block and maximal induction of maturing cells.

At 3.3 × 10^{-10} M TPA, some increase in the fraction of cells in the G2-M compartment occurred, and, at higher concentrations, >40% of the tetraploid population was present in this compart-
The accumulation in G2-M did not appear to be due to cell-cell adhesion, since cell disaggregation did not affect the magnitude of the G2-M peak (data not shown). It did not appear to derive from cell fusion because the percentage of binucleate cells was less than 6%, while accumulation in G2-M exceeded 40%. The accumulation in G2-M seen on Day 1 was retained on Day 3 (Chart 4C), at which time >80% of cells had entered the differentiation sequence, and >40% of tetraploid cells were located in the G2-M compartment.

**Effects of Combinations of Antineoplastic Agents and Conditioned Medium.** The effect of simultaneous administration of optimal concentrations of antineoplastic agents and conditioned medium is shown in Table 3. Additive or greater than additive (synergistic) effects were observed when antineoplastic agents were combined with conditioned medium, the fraction of maturing cells equalling or exceeding the value expected for an additive interaction.

Ara-C in combination with conditioned medium provided for an approximately additive drug effect. Maturing cells numbered 70% in cultures treated with the 2 agents, as compared to the calculated additive value of 67%. An additive interaction was also demonstrated by a functional test, the observed 40% NBT-positive cells coinciding with the expected value of 36%.

Daunorubicin and actinomycin D appeared to interact with conditioned medium in a greater than additive fashion. With a combination of daunorubicin and conditioned medium, 85% maturing cells were observed, as compared to an expected additive value of 67%. Similarly, while an additive interaction would have been expected to yield 35% NBT-positive cells, 63% NBT-positive cells were observed. In general, cell viability in cultures treated with 2 agents was similar to that seen in cultures treated with the antineoplastic agents alone.

The extent of differentiation induced by combinations of antineoplastic agents and conditioned medium approached that obtained with TPA. Approximately 69 to 85% of cells treated with antineoplastic drug:conditioned medium combinations displayed maturing phenotype on Day 3, as compared to ≥74% of cells treated with TPA. Likewise, about 78 to 87% of the cells treated with the combinations were viable, as compared to 83% in TPA-treated cultures. In cultures treated with conditioned medium:drug combinations, the fraction of NBT-positive cells was higher than in TPA-treated cultures, probably because TPA-treated cells exhibit a diminished NBT response under conditions of continuous drug exposure (data not shown). In contrast, the fraction of adherent cells was higher in TPA-treated cultures than in cultures treated with combinations of conditioned medium and antineoplastic agents.

**DISCUSSION**

Past studies have differed on the role DNA synthesis may play in the differentiation process (12, 13, 22, 30, 31, 33, 38, 40, 44). The studies described in this paper demonstrate that the nature of the relationship between DNA synthesis and differentiation depends upon the type of agent used. With antineoplastic agents as inducers, ML-1 cell differentiation follows extensive and sustained inhibition of DNA synthesis. In contrast, with conditioned medium and retinoic acid, initiation of differentiation precedes the cessation of DNA synthesis. Thus, inhibition of DNA synthesis is an initial event in differentiation induction with antineoplastic agents, in contrast to the pattern seen with the other agents, where a gradual decrease in DNA synthesis accompanies the maturation process.

These observations are compatible with a scheme (2) that...
allows for a drug- or factor-induced shift from the proliferation to the differentiation program. The finding that different inducers have different effects on DNA synthesis suggests that maturation may be induced by several mechanisms, one involving inhibition of the proliferation path and others entailing the direct activation of differentiation paths. Interference with the proliferation program by antineoplastic agents may entail modification of gene expression, whereas direct stimulation of the differentiation program may be brought about by modulation of growth and differentiation signals transmitted via the cell membrane.

While DNA synthesis does not appear to be required for maturation, RNA and protein synthesis seem necessary for implementation of the differentiation program. Upon treatment with inducing agents, RNA and protein synthesis are maintained at high levels at early time points and continue to various extents throughout the differentiation process. When RNA or protein synthesis is inhibited at early time points, as with cordycepin or cycloheximide, differentiation does not occur.

The mechanistic differences in the actions of different inducers are also demonstrated by the results of cell cycle analyses. Daunorubicin and actinomycin D rapidly block entry of ML-1 cells into S phase, in accord with the early inhibition of DNA synthesis. With conditioned medium and retinoic acid, some cell cycling continues at early time points, and accumulation in G₂ occurs in a progressive manner. This initial cycling may reflect proliferation of cells at various intermediate maturation stages, as occurs under in vivo conditions (6, 17).

Unlike the other antineoplastic differentiation inducers, ara-C, at early time points, causes a substantial fraction of cells to accumulate in S phase. As these cells disappear, some dead cells emerge. This observation suggests that S-phase cells may be sensitive to the cytotoxic effects of this drug, cells in other phases being capable of moving to differentiation. Thus, the cell cycle specificity of an agent may, in part, determine the extent of its cytotoxic versus differentiation-inducing effects.

Whereas prolonged exposure of the cells to these diverse agents or to moderate concentrations of TPA causes them to accumulate largely in G₁, a significant fraction of ML-1 cells exposed to high doses of TPA displays a G₂-M DNA content. These cells also appear capable of differentiation, as described for some other cell types in G₂-M phase (14, 52).

Taken together, the findings reported in this paper allow differentiation-inducing agents to be classified according to the effects they exert on DNA synthesis in relation to maturation induction. Class I comprises the antineoplastic agents which cause inhibition of DNA synthesis prior to the appearance of differentiation-associated characteristics. Inhibition of the proliferative path may be a primary effect of these drugs. Conditioned medium and retinoic acid belong to Class II, which includes agents that produce a substantial decline in DNA synthesis after the initiation of differentiation. Class III is represented by TPA which has some characteristics of both Class I and Class II agents, the decline in DNA synthesis and the initiation of differentiation occurring in an overlapping time frame. The fact that combinations of Class I and Class II agents provide additive or synergistic effects is compatible with the view that Class I and Class II agents act at separate sites, suggested to involve the proliferation and differentiation paths.

Class I and Class II agents display differences in the time course with which they induce maturation and loss of proliferative capacity. Generally, the onset of differentiation occurs rapidly after exposure to the Class II agents, being somewhat delayed with Class I agents. Conversely, Class I agents cause a rapid inhibition of proliferative capacity, while Class II agents allow some retention of proliferative capacity even after prolonged exposure.

The fact that differentiation-inducing antineoplastic agents, characterized by selective inhibition of DNA synthesis, can act additively or synergistically with natural inducing factors would appear to provide an optimal modality for therapy based on induced cancer cell maturation. This approach may offer an alternative to cytotoxic regimen.

ACKNOWLEDGMENTS

We thank John J. Maue, Rosemary A. Hromchak, Judith Anticola Leasure, and Pauline Sorets for their excellent technical assistance.

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