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Terminal differentiation of human promyelocytic leukemia cells induced by dimethyl sulfoxide and other polar compounds

(myeloid differentiation/liquid suspension culture/granulocyte/hemopoiesis)

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ABSTRACT **A human leukemic cell line (designated HL-60) has recently been established from the peripheral blood leukocytes of a patient with acute promyelocytic leukemia. This cell line displays distinct morphological and histochemical commitment towards myeloid differentiation. The cultured cells are predominantly promyelocytes, but the addition of dimethyl sulfoxide to the culture induces them to differentiate into myelocytes, metamyelocytes, and banded and segmented neutrophils. All 150 clones developed from the HL-60 culture show similar morphological differentiation in the presence of dimethyl sulfoxide. Unlike the morphologically immature promyelocytes, the dimethyl sulfoxide-induced mature cells exhibit functional maturity as exemplified by phagocytic activity. A number of other compounds previously shown to induce erythroid differentiation of mouse erythroleukemia (Friend) cells can induce analogous maturation of the myeloid HL-60 cells. The marked similarity in behavior of HL-60 cells and Friend cells in the presence of these inducing agents suggests that similar molecular mechanisms are involved in the induction of differentiation of these human myeloid and murine erythroid leukemic cells.**

Mouse erythroleukemia (Friend) cells have been extensively studied as a model for mammalian cell differentiation since the description of the erythroid differentiation-inducing effects of dimethyl sulfoxide (Me_2SO) upon these cells (1). The morphological, biochemical, and immunological changes induced by Me_2SO in Friend cells have been well characterized (1–3), but the precise mechanism of action of Me_2SO remains unknown. Similar changes have been induced by compounds other than Me_2SO, including a variety of low molecular weight, basic, highly polar compounds (4–6) as well as butyric acid (7). The inducing effects of Me_2SO are not limited to Friend cells but have been demonstrated in other rodent cell lines as well, including morphological and electrophysical changes in a mouse neuroblastoma cell line (8), lysozyme production in certain murine myeloid leukemic cells (9), and erythroid differentiation in a 7,12-dimethylbenz[*a*]anthracene-induced rat erythroleukemia (10).

We have recently developed a unique human cell line (designated HL-60) from the peripheral blood leukocytes of a patient with acute promyelocytic leukemia. These cultured cells display distinct morphological and histochemical myeloid characteristics (11). Unlike other human leukemic cell lines, which are primarily lymphoblastoid, virtually 100% of the cultured HL-60 cells are stained by myeloid-specific histochemical stains, including chloroacetate esterase, Sudan black, and myeloperoxidase. The majority of cells in this culture are promyelocytes. However, a significant percentage of cells (10–12%) are more mature, having morphological characteristics of myelocytes, metamyelocytes, and banded and segmented neutrophils. Although conditioned medium from a whole human embryo fibroblast strain was apparently required to initiate growth of the HL-60 cells (11), these cells, unlike human myeloid cells in our previous experience (12–14), do not require exogenous factor or conditioned medium for sustained growth. The cells have continuously proliferated and maintained distinct myeloid characteristics for over a year in suspension culture. Their morphological and histochemical myeloid characteristics are similar to those of the patient's uncultured leukemic blood cells, and the modal chromosome number of 44 found in the early passages of the HL-60 culture is the same as the modal chromosome number observed in fresh bone marrow obtained from the patient prior to chemotherapy (11). These findings, together with the ability of the cultured HL-60 cells to be tumorigenic in athymic *nude* mice (to be published elsewhere), indicate that the HL-60 cells are leukemic in origin.

In the present study we show that after the addition of Me_2SO and other polar compounds capable of inducing maturation in the above-mentioned rodent cell lines, there is a marked increase in the proportion of mature myeloid cells in the human HL-60 culture. Moreover, these morphologically mature cells, unlike the immature promyelocytes, are now capable of phagocytosis. Thus a differentiation-inducing effect of Me_2SO and related compounds has now been demonstrated in human cells. The fact that these compounds can induce differentiation in such a variety of different rodent and human cell lines suggests that they may trigger differentiation through common mechanisms in a wide range of mammalian cells.

MATERIALS AND METHODS

Cells. The HL-60 cells used in these experiments have been maintained in continuous suspension culture for over 1 yr for 48 passages. Cells are cultured in T/30 plastic flasks (Falcon Laboratories, Oxnard, CA) in RPMI-1640 medium (GIBCO, Grand Island, NY) supplemented with 20% heat-inactivated fetal calf serum (Flow Laboratories, Rockville, MD) and gentamicin at 50 μg/ml as detailed elsewhere (11). Cell growth was determined by trypan blue dye exclusion. For morphological assessment of the cells, Cytospin slide preparations of 0.2-ml aliquots of cell suspensions were prepared using a Shandon–Elliott Cytospin centrifuge (model SCA-0030, Surrey, England) and stained with Wright–Giemsa. Differential counts were then performed under light microscopy on a minimum of 200 cells for each experimental point.

Chemicals. Me_2SO was diluted from a 15% (vol/vol) stock solution of cryoprotective medium (Microbiological Associates, Bethesda, MD). All other reagents were purchased from Aldrich Chemicals (Milwaukee, WI).

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Abbreviations: Me_2SO, dimethyl sulfoxide; CSF, colony-stimulating factor.

DNA Synthesis. After variable exposure to Me_2SO, cells were washed free of Me_2SO and reincubated at a concentration of 2.5×10^5 cells per ml for 30 min at 37° in the presence of [^{3}H]thymidine (specific activity 0.36 Ci/mmol) at 10 μCi/ml. Five-milliliter cell suspensions were then collected, washed in cold phosphate-buffered saline, and precipitated with 10% trichloroacetic acid. The precipitate was collected on glass Millipore filters and washed with 5% trichloroacetic acid and then with 70% (vol/vol) ethanol. Radioactivity of the precipitate was then determined in Aquasol (New England Nuclear, Boston, MA) in a liquid scintillation counter.

Cloning of HL-60 Cells. HL-60 cells were suspended at concentrations of 10^4–10^6 cells per ml in RPMI-1640 medium containing 15% fetal calf serum and 0.8% methylcellulose, and 1 ml of the suspension was incubated in 35-mm petri dishes in a humidified 5% CO_2 environment at 37°. After 7–10 days, visible colonies containing 50–100 cells, presumably arising from a single cell, were individually transferred by sterile pasteur pipette to wells of a Linbro dish (Linbro Chemical Company, New Haven, CT) containing RPMI-1640 medium supplemented with 20% fetal calf serum. As growth continued, cells were transferred to larger wells and ultimately to Falcon T/30 flasks.

Phagocytosis. After exposure to various concentrations of Me_2SO, the cells were washed free of Me_2SO and resuspended at a concentration of 10^6 cells per ml in RPMI-1640 medium supplemented with 10% fetal calf serum and 10% human AB serum. *Candida albicans*, grown overnight in Sabouraud medium (Difco, Detroit, MI), were washed twice with saline and added to the above HL-60 cell suspension at a final concentration of 4×10^6 organisms per ml. The suspension was then incubated for 30 min at 37° and the percent of cells that had phagocytosed the fungi was determined by light microscopy on Wright–Giemsa stained preparations.

RESULTS

Changes in Morphology of HL-60 Cells Induced by Me_2SO. The morphological changes induced by Me_2SO in the HL-60 culture are illustrated in Fig. 1. In the absence of Me_2SO the HL-60 cells are predominantly typical promyelocytes (Fig. 1 *top*) with large round nuclei, each containing 2–4 nucleoli and dispersed nuclear chromatin. The cytoplasm is basophilic with prominent azurophilic granules, and the nuclear/cytoplasmic ratio is relatively high. Approximately 10–12% of the uninduced cells are more mature myeloid cells, predominantly myelocytes and metamyelocytes, with rare fully mature segmented neutrophils (Table 1). The addition of appropriate concentrations of Me_2SO to the culture induces in the majority of cells a striking morphological change characteristic of terminal differentiation of myeloid cells. The induced cells exhibit the following changes: smaller size, decreased nuclear/cytoplasmic ratio, less prominent cytoplasmic granules, marked reduction or complete disappearance of nucleoli, pyknotic changes in nuclear chromatin, and marked indentation, convolution, and segmentation of the nuclei (Fig. 1 *middle*). Metamyelocytes and neutrophilic bands predominate in the culture 5–7 days after induction rather than segmented neutrophils, the final cells in the myeloid series (Table 1). This pattern is analogous to that seen in induced Friend mouse erythroleukemia cells in which complete erythroid differentiation with extrusion of nuclei and progression to the most mature red blood cells is infrequent even after incubation with the most potent inducing agents (5).

Changes in Cell Growth Kinetics of HL-60 Cells Induced by Me_2SO. Growth of HL-60 cells begins to plateau at day 5 after treatment with Me_2SO concentrations of 1.25% and below

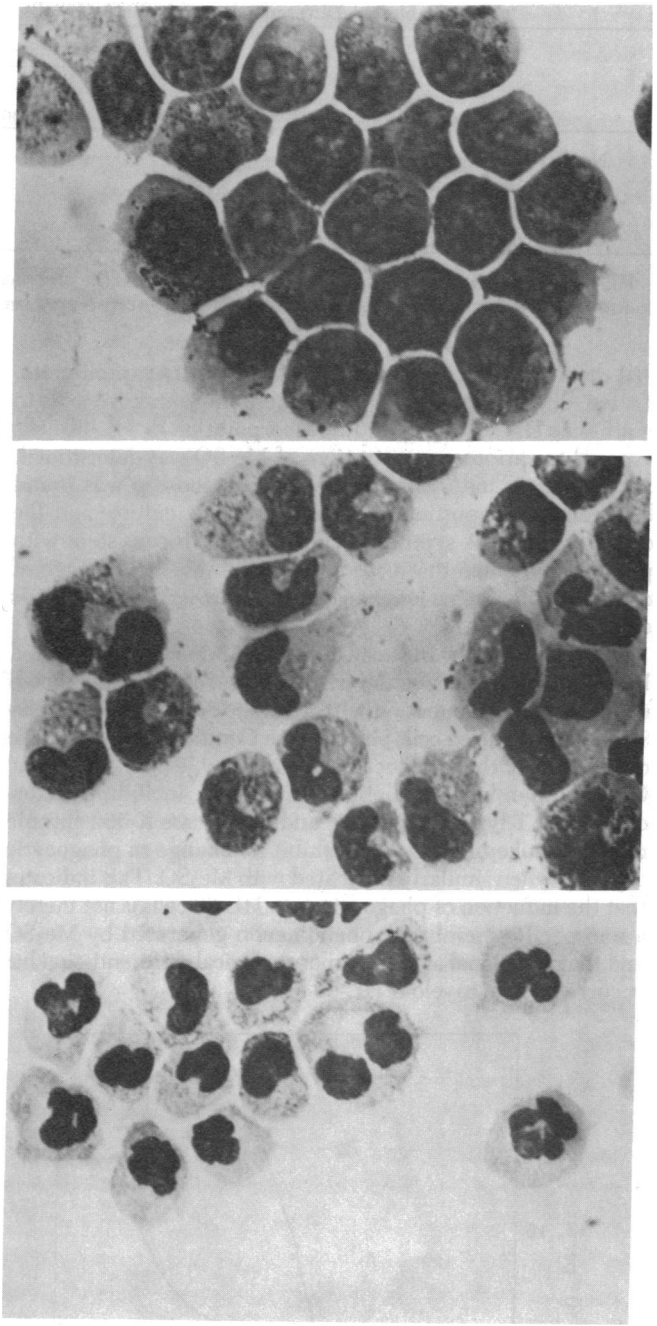

FIG. 1. Morphological appearance of uninduced and induced HL-60 cells. Cytospin preparations of suspension cell cultures were stained with Wright–Giemsa before and after treatment with inducing compounds. (*Top*) HL-60 cells cultured without inducer. (×660.) (*Middle*) Cells cultured in 1.25% Me_2SO for 6 days. (×660.) (*Bottom*) Cells cultured in 60 mM dimethylformamide for 6 days. (×500.)

(Fig. 2). Concentrations of 1.5% or higher completely inhibit cell growth. Maximum differentiation occurs 5–7 days after addition of Me_2SO (Fig. 3). The concentration of Me_2SO that gives maximum induction of maturation without inhibition of cell replication is approximately 1.3%. These findings are remarkably similar to those described for Me_2SO-induced maturation of Friend mouse erythroleukemia cells (1), except that the concentrations of Me_2SO required for differentiation and inhibition of cell division are slightly lower in the HL-60 system.

The plateaus in the growth curves at 5 days (Fig. 2) suggest

Table 1. Differential counts of HL-60 cells after incubation with various inducing compounds

Inducer	Myeloid cell type, % of total cells					
	Myeloblasts	Promyelocytes	Myelocytes	Metamyelocytes	Banded neutrophils	Segmented neutrophils
None	13	76	7	3	1	0
Dimethylformamide (60 mM)	1	14	16	42	17	10
Me_2SO (1.25%)	0	17	17	38	26	2
Butyric acid (0.6 mM)	0	42	44	14	0	0

HL-60 cells were incubated under standard conditions in the presence of the indicated concentrations of inducing agent. After 6 days, differential counts were performed on Wright–Giemsa stained Cytospin preparations of the cell suspensions.

that the mature cells no longer have the capacity to proliferate. To test directly the capability of these more mature cells to synthesize DNA, [^{3}H]thymidine incorporation by HL-60 cells exposed to various concentrations of Me_2SO was determined. Six days after induction, an inverse relationship was found between the proportion of mature cells in a culture and the capacity for DNA synthesis (Table 2). This is consistent with previous evidence that cells beyond the myelocyte stage of differentiation are no longer capable of further DNA synthesis and cell division (15).

Phagocytosis by Induced Cells. Uninduced and induced HL-60 cells after incubation with *Candida albicans* are shown in Fig. 4. After incubation with 1.25% Me_2SO for 7 days, 85–90% of the cultured cells phagocytose *Candida albicans*, while only 5–10% of the uninduced cells are capable of phagocytosis. Other human hematopoietic cultured cells, including factor-dependent T lymphocytes (16) and blast phase K-562 chronic myeloid leukemic cells (17) exhibit no change in phagocytic capacity when similarly incubated with Me_2SO. This indicates that the induction of phagocytosis in HL-60 cells is not merely a nonspecific membrane phenomenon generated by Me_2SO and that functional as well as morphological differentiation has occurred in the myeloid HL-60 cells.

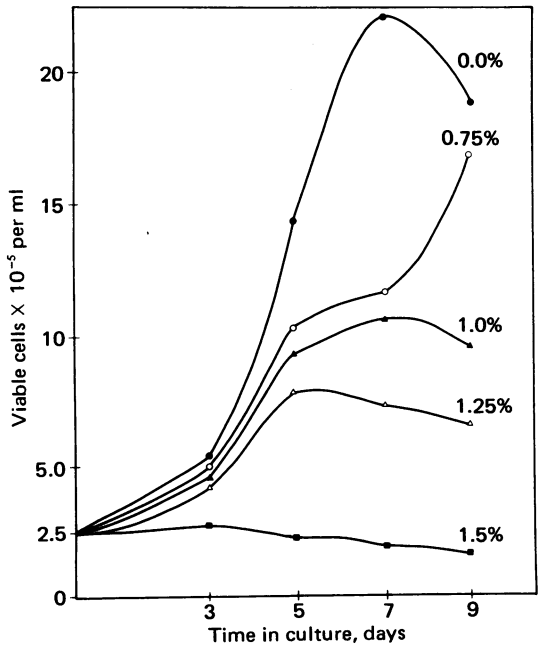

FIG. 2. Growth of HL-60 cells in various concentrations of Me_2SO. Cells were seeded at a concentration of 2.5 × 10^5 cells per ml on day 0 and incubated under standard conditions in the indicated concentrations of Me_2SO. On the days noted, cells were counted in a hemocytometer chamber. Viability was determined by trypan blue dye exclusions.

Induction of HL-60 Differentiation by Compounds Other than Me_2SO. Although Me_2SO has been the most widely studied inducing compound, numerous other low molecular weight, polar, freely diffusible compounds exist which induce differentiation of Friend mouse erythroleukemia cells at molar concentrations significantly less than that required for Me_2SO (4–6). We have tested some of these compounds, and all those tested also induce myeloid differentiation of the HL-60 cells (Table 3). The kinetics of induction and extent of morphological differentiation (Fig. 1 *bottom*) are similar to those described above for Me_2SO. Although the optimal molar concentrations of the polar inducers in the HL-60 system are 40–60% less than the concentrations used in the Friend mouse cell system (4), the relative potency of the compounds in inducing differentiation is the same in both systems. These striking similarities in the behavior of Friend cells and HL-60 cells in the presence of these compounds suggest that similar mechanisms may be involved in the terminal differentiation of both.

Butyric acid, a potent inducer of differentiation in Friend cells (7) also induces differentiation of the myeloid HL-60 cells at an optimal concentration of 0.6 mM, a concentration con-

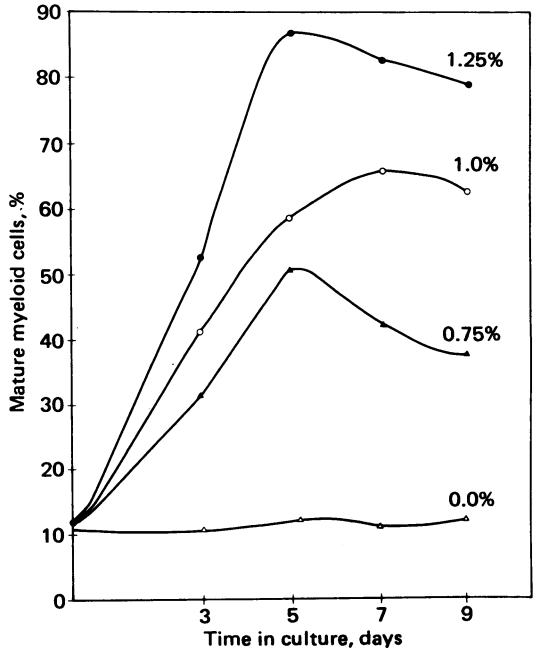

FIG. 3. Differentiation induced in HL-60 cells by various concentrations of Me_2SO. Cells were seeded at a concentration of 2.5 × 10^5 cells per ml on day 0 and incubated under standard conditions in the indicated concentrations of Me_2SO. At the intervals noted, differential counts were performed on Wright–Giemsa stained Cytospin preparations of 0.2-ml aliquots of the cell suspensions. "Mature myeloid cells" refers to myelocytes, metamyelocytes, and banded and segmented neutrophils.

Table 2. Effect of Me_2SO induction on DNA synthesis of HL-60 cells

Me_2SO concentration, %	Mature myeloid cells,* %	[^{3}H]Thymidine incorporation, cpm/10^6 viable cells
0	13	7258
1.00	58	3983
1.15	73	1609
1.30	94	714

Cells were incubated under standard conditions with the indicated concentrations of Me_2SO. After 6 days of incubation, cells were washed free of Me_2SO and [^{3}H]thymidine incorporation into trichloroacetic acid-precipitable material was determined.

* Myelocytes, metamyelocytes, and banded and segmented neutrophils on Wright–Giemsa-stained Cytospin preparations of cell suspensions after 6 days of incubation with Me_2SO.

siderably less than that required for the above polar compounds. The extent of morphological differentiation with butyric acid is more limited with myelocytes predominating in the induced HL-60 culture (Table 1). Isobutyric acid failed to induce differentiation of HL-60 cells, as has also been noted with Friend cells (7).

Response of Clones of HL-60 Cells to Me_2SO. We have developed 150 clones of HL-60 cells using the cloning technique in semi-solid medium as described in *Materials and Methods*. All of these clones manifest a response to Me_2SO similar to that shown in Figs. 1, 2, and 3, with some clones developing over 98% mature myeloid cells after 5–7 days of incubation with inducer.

DISCUSSION

The properties of the HL-60 cells before and after induction of maturation with Me_2SO are summarized in Table 4. All of the observed alterations are characteristic of the differentiation of promyelocytes to mature granulocytes. However, additional detailed morphological, ultrastructural, biochemical and functional analyses are required to determine if the HL-60 promyelocytes and their more mature progeny differ from normal myeloid cells.

Because acute myeloid leukemia can be looked upon as a disease primarily involving a block in myeloid differentiation (18) and continues to be the most difficult leukemia to treat (19), therapeutic intervention using compounds capable of removing such a block in differentiation might be suggested by these results. However, several considerations must temper this ap-

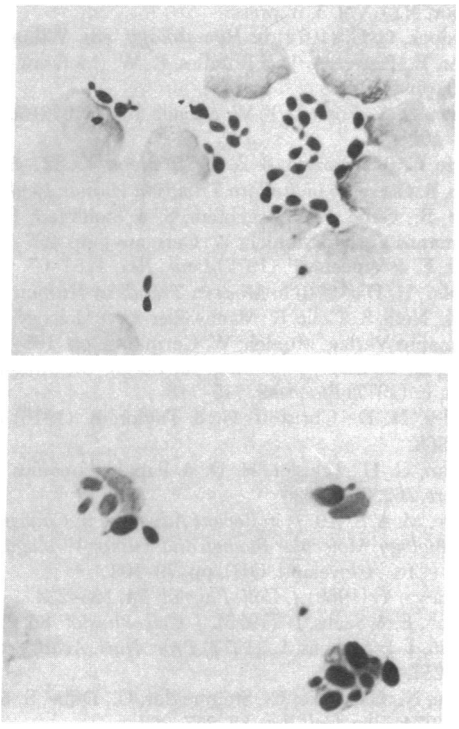

FIG. 4. Phagocytosis of *Candida albicans* by uninduced and induced HL-60 cells. HL-60 cells incubated with *Candida albicans* were Wright–Giemsa stained as detailed in the text. (×500.) (*Upper*) Uninduced HL-60 cells incubated with *Candida*. (*Lower*) Induced HL-60 cells incubated with *Candida*.

Table 3. Polar compounds other than Me_2SO active in inducing terminal differentiation of HL-60 cells

Inducer	Optimal concentration, mM	Mature myeloid cells,* %
Acetamide	150	71
N-Methylacetamide	20	68
N,N-Dimethylacetamide	10	73
N-Methylformamide	150	81
N,N-Dimethylformamide	60	84
Piperidone	37.5	78
1-Methyl-2-piperidone	4	65
Triethylene glycol	100	60

Differential counts were performed on Wright–Giemsa stained Cytospin preparations of HL-60 cells after 6 days of incubation with the indicated concentration of inducer.

* Myelocytes, metamyelocytes, and banded and segmented neutrophils.

Table 4. Characteristics of uninduced and induced HL-60 cells

Characteristic	Uninduced HL-60	Induced HL-60*
Morphological		
Nucleus	Round with dispersed chromatin	Pyknotic
Nucleoli	2–4 per nucleus	Usually absent
Cytoplasm	Deeply basophilic Prominent granules	Pinkish hue Granules less prominent
Nuclear/cytoplasmic ratio	High	Low
Histochemical		
Chloroacetate-esterase	>98% positive	>98% positive
Sudan black	>98% positive	>98% positive
Myeloperoxidase	>98% positive	>98% positive
Biochemical		
[^{3}H]Thymidine incorporation†	7258 cpm/10^6 cells	714 cpm/10^6 cells
Physiological		
Phagocytosis (% capable)‡	<10%	>85%
Growth in suspension	Continuous proliferation for >1 year	Proliferation ceases

* Cells were induced by incubation for 6 days under standard conditions in 1.3% Me_2SO.

† [^{3}H]Thymidine incorporation into trichloroacetic acid-precipitable material.

‡ Capability of phagocytosing *Candida albicans*.

proach. With the exception of butyric acid, the compounds used in these experiments are organic solvents that undoubtedly would have many toxic effects when used in the concentrations described here. Indeed, initial experiments in mice using *N*-methylacetamide to treat Friend erythroleukemia were complicated by the hepatotoxicity of this compound (20). Moreover, both Friend cells and HL-60 cells are unusual among leukemic cells in their clear commitment to differentiate. In most other leukemic cells the putative block in differentiation occurs at an earlier stage, with the commitment to differentiation being less defined, and Me_2SO may not have a similar inducing effect on these less committed cells. For example, the K-562 cell line, derived from a patient with chronic myelogenous leukemia in blast crisis (17), contains myeloid-specific membrane antigens (21) but exhibits no morphological or histochemical myeloid differentiation and hence represents a cell line with an earlier arrest in myeloid differentiation than the HL-60 cells. We have not been able to induce morphological or histochemical changes in the K-562 cells with the addition of Me_2SO.

The mechanism of action of Me_2SO and other polar compounds in inducing terminal differentiation of Friend cells and HL-60 cells remains unknown. Most of the inducing compounds have cryoprotective properties (22). This has led to the suggestion that their primary mechanism of action may involve alteration of cell membranes (23). Others have suggested that these freely diffusible compounds may act directly in the nucleus, somehow altering conformation of DNA–chromatin complexes in such a way as to initiate transcription of genes coding for differentiation (4). The similarity in the behavior of Friend mouse erythroleukemia cells and HL-60 cells in the presence of these compounds suggests that similar fundamental biological mechanisms are involved in inducing differentiation in both systems. Hence, the myeloid HL-60 cells provide another convenient cell system for studying the mechanism of action of these differentiation-inducing compounds. Moreover, the HL-60 culture provides unlimited quantities of human promyelocytes for biochemical and immunological studies as well as a unique system for the study of human myeloid differentiation in liquid suspension.

The ability of both HL-60 and Friend leukemia cells to undergo maturation under appropriate *in vitro* conditions indicates, that despite karyotypic abnormalities, the genetic information coding for terminal differentiation in these cells is present and capable of being phenotypically expressed. The reason why such genes are not expressed in the leukemic host is presently unknown, but may be related to abnormalities in host differentiation-inducing factors that normally trigger committed stem cells to differentiate, or to abnormal stem cell receptors for such factors (24). Certain mouse myeloid cell lines, derived from mice with myeloid leukemia, undergo normal morphological and functional maturation *in vitro* when induced by colony-stimulating factor (CSF), the presumed normal inducer of myeloid differentiation (25–27). The absence of such normal gene expression *in vivo* could be explained by a blockage or alteration of the normal CSF receptors on these leukemic cells *in vivo* or by defective CSF production by the leukemic host. Alternatively, receptors for normal host differentiation-inducing factors could be defective or absent on certain other leukemic cells. For example, Friend mouse erythroleukemia cells, unlike the above mouse myeloid cells, do not respond *in vitro* to erythropoietin (28), the presumed normal inducer of erythroid differentiation, suggesting an absent or defective receptor for this molecule on the surface of Friend cells. This could account for the failure of Friend cells to differentiate *in vivo* even in the presence of normal host erythropoietin. Characterizing the response of the HL-60 cells to various sources of CSF may determine whether normal or defective CSF receptors exist on their surfaces.

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